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Isolation and Identification of Betulin, Lupeol, and β-Amyrin from the Bird-lime of *Trochodendron aralioides* Siebold et Zuccarini*

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Received September 19, 1956

Three triterpenoid alcohols: betulin, lupeol, and β-amyrin have now been isolated from the unsaponifiable fraction of the ethereal extract of the bark of *Trochodendron aralioides* Siebold et Zuccarini (*Trochodendraceae*). Of these, betulin has been further characterized as allobetulin. "*Trochosterin*"[1], "an alcohol of m.p. 210°"[2], and "an alcohol (acetate: m.p. 170°)"[3], described by earlier groups of workers, have now been confirmed to be nothing other than a mixture composed of lupeol and β-amyrin respectively.

Trochodendron aralioides Siebold et Zuccarini** (Japanese name: Yamaguruma; *Trochodendraceae*) is a tree indigenous to the southern parts of Japan, Loochoo, and Formosa. It is well-known that the bark of this plant contains an amount of resinous matter which is called "red bird-lime", and made use of ensnaring little birds, etc., in Japan.

In 1921, the isolation from the unsaponifiable fraction of "red bird-lime" of a compound, "trochol" of $C_{26}H_{44}O_2$ and m.p. 252° (acetate, m.p. 217°), was first reported by H. Yanagizawa[1].

Almost about the same time, the isolation from the same material of a compound, "trochodiol" of $C_{29}H_{50}O_2$ and m.p. 252° (acetate, m.p. 217°) was also reported by Y. Nishizawa[2].

Later, in 1942, the isolation from the same material of a triterpenoid diol of $C_{30}H_{50}O_2$ of m.p. 251°, (acetate, m.p. 217°), which was proved to be identical with betulin (m.p. 251°), (acetate, m.p. 217°) isolated from the bark of *Betula latifolia* Komarov (*Betulaceae*), was reported by K. Sisido et al[3].

On the basis of close similarity in their characteristic properties, it was concluded by K. Sisido et al.[3], that each of the aforementioned "*trochol*" and "*trochodiol*" must be identical with betulin, and hence, both of the names of "trochol" and "trochodiol" should not be used hereafter in the literature.

Besides betulin further isolation from the same material of: a) a monohydric alcohol named "*trochosterin*"[1] (A) of m.p. 172-175°, (acetate, m.p. 190°); b) an alcohol[2] (B) of m.p. 210°, (acetate, m.p. 190°); and c) an alcohol[3] (C), (acetate, m.p. 170°) was described by earlier groups of workers.

The present paper deals with the isolation and identification of betulin, lupeol and β-amyrin present in the unsaponifiable fraction of the ethereal extract of the bark of *Trochodendron aralioides* Siebold et Zuccarini.

In order to achieve satisfactory separation of the triterpenoids, the acetylated mixture of the unsaponifiable fraction was subjected to fractional crystallization, using ethanol as an appropriate solvent.

As was reported by K. Sisido et al.[3],

* Part III: This Bulletin, **20**, 97; 206 (1956). This is Part IV of a series of papers entitled as "Studies of Constituents of Various Sorts of Bird-lime"; Presented at the Meeting of the West Japan Branch of the Agricultural Chemical Society of Japan, held at May 12, 1956, at Kyushu University, Fukuoka, Japan.

** Siebold et Zuccarini, "*Flora Japonica*", **I**, 84 (1835-41).

1) H. Yanagizawa, *J. Pharm. Soc. Japan*, **41**, 405 (1921); H. Yanagizawa, N. Nakashima, *ibid.*, **42**, 179 (1922); **43**, 251 (1923).

2) Y. Nishizawa, *J. Chem. Soc. Tokyo*, **41**, 1043 (1920); *J. Chem. Soc. Japan*, **43**, 154; 810 (1922); **44**. 881 (1923).

3) K. Sisido, S. Narita, *J. Ind. Chem. Soc. Japan*, **45**, 1187 (1942).

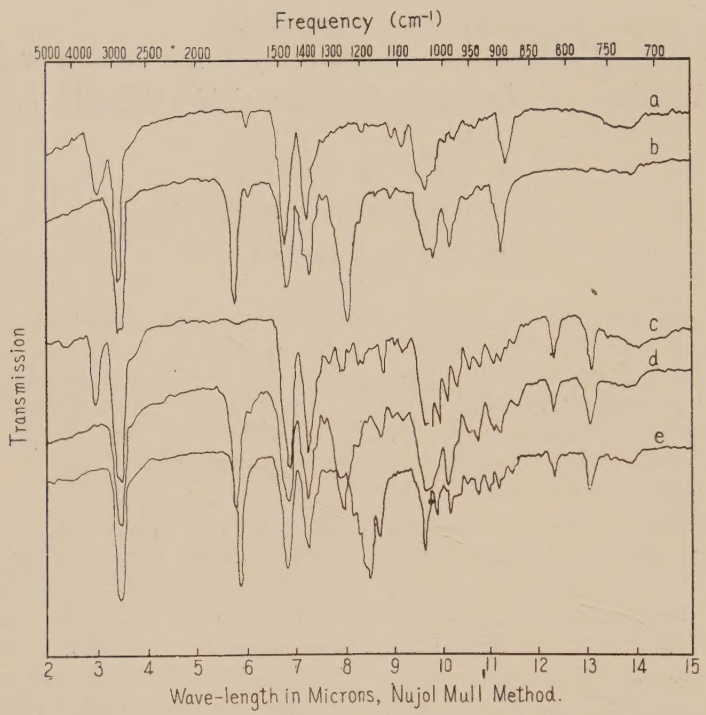

FIG. 1. Infrared Spectra of Betulin, alloBetulin, and Their Derivatives.
a): Betulin, b): Betulin Diacetate, c): alloBetulin, d): alloBetulin Acetate, and e): alloBetulin Formiate.

betulin diacetate was isolated from the fraction sparingly-soluble in ethanol, and was hydrolyzed to yield betulin, which was further identified by isomerizing it with formic acid into allobetulin formiate[4] of $C_{31}H_{50}O_3$, m.p. 313–315° and $[\alpha]_D^{30}+49.3°$.

It has now been found that treatment with formic acid of betulin diacetate gives rise to allobetulin acetate of $C_{32}H_{52}O_3$, m.p. 280–281°, and $[\alpha]_D^{30}+54.2°$. Both of these formiate and acetate of allobetulin were hydrolyzed to yield allobetulin of m.p. 264–265° and $[\alpha]_D^{30}+48.3°$ respectively.

In addition, it has now also been found that treatment with perchloric acid of betulin diacetate gives rise to allobetulin, indicating its simultaneous isomerisation and deacetylation.

As shown in Figure 1, the infrared spectra of betulin and its acetate reveal sharp bands 882 cm^{-1} and at 889 cm^{-1} respectively, characteristic of the grouping $\begin{matrix}R\\R\end{matrix}\!\!>\!C=CH_2$. The infrared spectra of allobetulin, its acetate and formiate reveal characteristic sharp bands at 814 and 768 cm^{-1}; at 811 and 767 cm^{-1}; and at 814 and 767 cm^{-1} respectively. A detailed interpretation of these spectra will be reported elsewhere later.

Deacetylation-benzoylation of the fraction freed of betulin diacetate afforded a mixture (m.p. 235–245°) of lupeol benzoate and β-amyrin benzoate, which was subjected to further fractional crystallization, using a mixture of ethyl-acetate and ethanol (1:1) as an appropriate solvent mixture.

From the fraction almost insoluble in this

4) H. Schulz, K. Pieroh, *Ber.*, **55**, 2332 (1922); L. Ruzicka, F. Govaert, N.W. Goldberg, A.H. Lamberton, *Helv. Chim. Acta*, **21**, 73 (1938); R. Kawaguchi, K. Kin, *J. Pharm. Soc. Japan*, **60**, 343 (1940). It seems most likely that "*trochol formiate*" of m.p. 306° described by H. Yanagizawa et al. (*J. Pharm. Soc. Japan*, **43**, 251 (1923), might be nothing other than *allo*betulin formiate.

solvent mixture, lupeol benzoate was isolated, and was debenzoylated to yield lupeol.

Furthermore, from the fraction freed of lupeol benzoate, β-amyrin benzoate was isolated, and was also debenzoylated to yield β-amyrin.

Noteworthy is the fact that satisfactory separation of a mixture of lupeol and β-amyrin could only be achieved by fractional crystallization of their benzoates from a mixture of ethyl-acetate and ethanol, and by their subsequent debenzoylation.

Now it follows from the foregoing that a mixture of triterpenoids obtained from the unsaponifiable of "red bird-lime", is composed of betulin, lupeol, and β-amyrin, capable of being fractionated into single entities, indicative of the fact that each of the aforementioned monohydric alcohols: (A)[1], (B)[2], and (C)[3] described by earlier groups of workers, are not single entities, but nothing other than a mixture of lupeol and β-amyrin respectively.

It is also of interest to point out the fact that β-amyrin has been shown to be present in the unsaponifiable fraction of all sorts of bird-lime hitherto examined[5], and the cooccurrence of lupeol and β-amyrin has been noted, e.g., in the unsaponifiable of bird-lime from the bark of *Ilex integra* Thunberg[5b] (*Aquifoliaceae*), and in the herbs of Japanese mistletoe[6] (Japanese name: Yadorigi; *Loranthaceae*), i.e., "*Viscum album* Linnaeus *var. coloratum* Ohwi".*

EXPERIMENTAL**

Isolation and Hydrolysis of the Resin (Bird-lime). After exhaustive extraction (40 hrs.) with ether of the chopped and dried bark (1.5 kg)***, there was obtained 264.0 g of orange-colored extract (17.60% of the dried bark), which was soluble in 10 times its weight of cold benzene.

After being refluxed with 10% benzene-ethanolic KOH-solution (benzene: ethanol 1:1) for 40 hrs., the resulting reaction mixture was filtered while hot. After removal of the solvents *in vacuo*, the residue was diluted with 2.5 l of water, and subjected to continuous extraction with ether for 45 hrs. The aqueous layer was combined with the washings, acidified with hydrochloric acid, and extracted with ether to yield a fatty-acid fraction (I) (132.5 g: 8.83%), whereby no separation of potassium salt of triterpenoid acid was observed between the alkaline and ethereal layer.

After drying and removal of ether, there was obtained a pale-yellow crystalline mass (129.5 g: 8.63%), which was boiled with 4~5 times its weight of ethanol for 1 hr., cooled, and filtered.

The resulting filtrate was concentrated to a small volume, and the concentrate was refluxed with a suitable amount of ethanol as described above. After repetition of the procedure, there were finally obtained: a) a yellowish, syrupy fraction (II) (57.0 g: 3.89%), which was readily dissolved in ethanol and became semisolid after being left for a week; and, b) colorless needles (III) of m.p. 225–235° (72.5 g: 4.83%).

Isolation of Betulin Diacetate. To a solution of 72.5 g of III in 150 ml of benzene was added 80 ml of acetic anhydride, and the mixture was refluxed for 2 hrs. After distillation *in vacuo*, the remaining crude acetate was washed with water, dried, and recrystallized from ethanol, giving: a less-soluble fraction (IIIa) of m.p. 213–218° (28.5 g), as prismatic needles, and, b) a more-soluble fraction (IIIb) of m.p. 190–193°, (44.0 g) as needles.

An amount of 28.5 g of IIIa was further recrystallized several times either from ethanol or a mixture of ethyl-acetate and ethanol (1:1) to yield prismatic needles (7.5 g), melting at 221.6–222.6°. $[\alpha]_D^{30}$ +21.4° (c, 1.064). (For infrared spectra data, see Fig. Ib.).

Anal. Found: C, 77.32; H, 10.40. Calcd. for $C_{34}H_{54}O_4$: C, 77.72; H, 10.33.

Betulin. Two grams of the above diacetate of m.p. 221.6–222.6° was refluxed with 5% ethanolic KOH-solution for 2 hrs.. After removal of ethanol, the residue was diluted with water, and extracted with ether. The ethereal layer was washed with

5) a) S. Iseda, *J. Pharm. Soc. Japan*, **72**, 1611 (1952); b) S. Iseda, K. Yagishita, N. Toya, *ibid.*, **74**, 422 (1954); c) K. Yagishita, This Bulletin, **20**, 97; 206 (1956); d) K. Yagishita, in press. The cooccurrence of α- and β-amyrin has been demonstrated in the unsaponifiable fraction of bird-lime from the bark of *Ilex latifolia* Thunberg (*Aquifoliaceae*).

6) Y. Obata, *J. Agr. Chem. Soc. Japan*, **17**, 222; 784 (1941).

* The valid scientific name of Japanese mistletoe.

** All melting points were corrected; Rotations were measured in $CHCl_3$ solution, using 1-dm tube. Infrared spectra were measured in Nujol with a Perkin Elmer 21 double-beam instrument, using sodium chloride prisms.

*** The bark used in this work was collected in Island Yakushima, Kagoshima Prefecture.

water, dried, and then distilled. The residue after recrystallizations thrice from ethanol afforded colorless prismatic needles (1.8 g), melting at 255.5–256.5°. $[\alpha]_D^{30}$ +16.2° (c, 0.832). (For infrared spectra data, see Fig. Ia).

Anal. Found: C, 78.77; H, 11.66. Calcd. for $C_{30}H_{50}O_2 \cdot C_2H_5OH$: C, 78.68; H, 11.47. Found (in material dried at 120°/5–7 mm for 3 days): C, 81.25; H, 11.53. Calcd. for $C_{30}H_{50}O_2$: C, 81.39; H, 11.38.

Isomerisation of Betulin into alloBetulin, Formation of alloBetulin Formiate. A mixture of 2.0 g of betulin of m.p. 255.5–256.5° and 20 ml of formic acid (98–100%) was refluxed for 45 min. After being left overnight, the reaction mixture was poured onto an amount of water, and the crude product separated was filtered, washed, and dried. It was recrystallized thrice from benzene, giving flat needles of m.p. 313–315° (1.5 g). $[\alpha]_D^{30}$ +49.3 (c, 0.540). (For infrared spectra data, see Fig. Ie).

Anal. Found: C, 79.54; H, 10.79. Calcd. for $C_{31}H_{50}O_3$: C, 79.10; H, 10.71.

alloBetulin. One gram of allobetulin formiate of m.p. 313–315° was refluxed with 5% ethanolic KOH-solution for 2 hrs. to yield crude allobetulin, which after recrystallizations thrice from a mixture of chloroform and methanol (1:1), afforded small triangular plates (0.9 g), melting at 264–265°. $[\alpha]_D^{30}$ +48.3° (c, 0.565). (For infrared spectra data, see Fig. Ic).

Anal. Found: C, 81.54; H, 11.45. Calcd. for $C_{30}H_{50}O_2$: C, 81.39; H, 11.38.

A mixture of this alcohol of m.p. 264–265° and betulin of m.p. 255.5–256.5° melted at 230–236°.

alloBetulin Acetate. To a solution of 0.6 g of allobetulin of m.p. 264–265° in 1 ml of pyridine, was added 1 ml of acetic anhydride, and the mixture was refluxed for 1 hr.. After being left overnight, the resulting reaction mixture was poured onto ice-cooled water, and the crude acetate separated was filtered, washed with water, and then dried. After recrystallizations thrice from a mixture of ethanol and benzene (1:1), the pure allobetulin acetate crystallized as hexagonal plates, and melted at 280–281°. $[\alpha]_D^{30}$ +54.2° (c, 0.656). Yield was 0.5 g. (For infrared spectra data, see Fig. Id).

Anal. Found: C, 79.25; H, 10.84. Calcd. for $C_{32}H_{52}O_3$: C, 79.28; H, 10.81.

Deacetylation of this acetate afforded allobetulin of m.p. 264–265°.

Conversion of Betulin Diacetate into alloBetulin Acetate. A mixture of 1.0 g of betulin diacetate of m.p. 221.6–222.6° and 15 ml of formic acid (98–100%), was refluxed for 1.5 hrs..

After being left for 1 hr., the resulting reaction mixture was poured onto an amount of water, and the crude product separated was filtered, washed with water, and then dried. The residue was dissolved in 50 ml of benzene, and after the solution was run down through a column of alumina (2×5 cm). The column was eluted with 50 ml of benzene, giving a fraction (0.8 g), which after several recrystallizations from a mixture of benzene and ethanol (1:1), yielded hexagonal plates, melting at 280–281° either alone or on admixture with the above specimen of allobetulin acetate. $[\alpha]_D^{30}$ +54.0° (c, 0.682).

Anal. Found: C, 79.22; H, 10.86. Calcd. for $C_{32}H_{53}O_3$: C, 79.28; H, 10.81.

Deacetylation of this acetate afforded monohydric alcohol, which after several recrystallizations from a mixture of chloroform and methanol (1:1), gave triangular plates of m.p. 264–265°, proved to be identical with the above specimen of allobetulin (mixed m.p. and infrared spectra). $[\alpha]_D^{30}$ +48.4° (c, 0.625).

Treatment with Perchloric Acid of Betulin Diacetate. To a solution of 0.5 g of betulin diacetate in 10 ml of benzene was added a mixture of 3 ml of perchloric acid (60%) and 50 ml of ethanol, and the mixture was refluxed for 2 hrs.. After being left for 2 hrs., the resulting reaction mixture was poured onto ice-cooled water. There separated a colorless mass, which was washed with water, and dried. The residue was dissolved in 30 ml of benzene, and the solution was run through a column of alumina (2×4 cm). The column was eluted with 50 ml of benzene to yield a fraction (0.42 g), which after several recrystallizations from ethanol afforded triangular plates of m.p. 265–266°, proved to be identical with the above specimen of allobetulin (mixed m.p. and infrared spectra). $[\alpha]_D^{30}$ +48.5° (c, 0.663).

Anal. Found: C, 81.58; H, 11.58. Calcd. for $C_{30}H_{50}O_2$: C, 81.39; H, 11.38.

Isolation of Lupeol Benzoate. An amount of 44.0 g of IIIb of m.p. 190–193° was recrystallized 6 times from ethanol to yield colorless microneedles of m.p. 215–217°, which were shown to be composed of mixed entities. Deacetylation-debenzoylation of IIIb afforded a mixture of benzoates of m.p. 235–245°, which was recrystallized thrice from 80~100 times its weight of a mixture of ethyl-acetate and ethanol (1:1).

From the fraction almost insoluble in this solvent,

there was obtained benzoate in leaflets, melting at 269–270°, proved to be identical with an authentic specimen of lupeol benzoate[4a,b] of m.p. 269–270°. (mixed m.p. and infrared spectra). $[\alpha]_D^{21}$+53.8° (c, 1.052).

Anal. Found: C, 83.69; H, 10.15. Calcd. for $C_{37}H_{54}O_2$: C, 83.71; H, 10.25.

Lupeol. Three grams of lupeol benzoate of m.p. 269–270° was refluxed with 5% ethanolic KOH-solution for 2 hrs.. After removal of ethanol, the residue was diluted with water, and extracted with ether. The ethereal layer was dried, and distilled. After recrystallizations thrice from ethanol of the residue, there was obtained lupeol in fine needles (2.7 g), melting at 215–216° either alone or on admixture with an authentic specimen[5a]. $[\alpha]_D^{30}$+28.0° (c, 0.721). The infrared spectrum of this substance was found to be superimposable with that of an authentic specimen[5a].

Anal. Found: C, 84.28; H, 11.97. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

Lupeol Acetate. One and a half grams of lupeol of m.p. 215–216° in 15 ml of benzene, was acetylated with 3 ml of acetic anhydride.

After removal of the solvents *in vacuo*, the residue was poured onto an amount of ice-cooled water, and the crude acetate separated was filtered, washed with water, and then dried. After recrystallizations thrice from ethanol, the pure acetate was obtained in needles (1.4 g), melting at 217–218°. $[\alpha]_D^{30}$+44.6° (c, 0.551).

Anal. Found: C, 81.80; H, 11.09. Calcd. for $C_{32}H_{52}O_2$: C, 81.99; H, 11.18.

Deacetylation of this acetate afforded lupeol of m.p. 215–216°.

Isolation of β-Amyrin Benzoate. From the fraction freed of lupeol benzoate, there was obtained crude benzoate, which after several recrystallizations from a mixture of acetone and benzene (1:1), afforded colorless plates, melting at 232° either alone or on admixture with an authentic specimen of β-amyrin benzoate[5c] of m.p. 232°. $[\alpha]_D^{28}$+97.4° (c, 1.960). Yield was 1.5 g.

Anal. Found: C, 83.52; H, 10.52. Calcd. for $C_{37}H_{54}O_2$: C, 83.71; H, 10.25.

Isolation of β-Amyrin Acetate. To a solution of 57 g of II in 250 ml of benzene was added 60 ml of acetic anhydride, and the mixture was refluxed for 3 hrs.. After removal of the solvents *in vacuo*, the remaining crude acetate was dissolved in 250 ml of glacical acetic acid, and left in the refrigerator for several days. There separated scales, which were filtered, washed with water, dried, and recrystallized several times from ethanol. The pure acetate crystallized as prismatic needles (12.5 g), and melted at 239–240° either alone on admixture with an authentic specimen[5c]. $[\alpha]_D^{28}$+82.6° (c, 0.734).

Anal. Found: C, 82.02; H, 11.35. Calcd. for $C_{32}H_{56}O_2$: C, 81.99; H, 11.18.

β-Amyrin. a) Half a gram of β-amyrin benzoate of m.p. 232° was refluxed with 5% ethanolic KOH-solution for 2 hrs. to yield β-amyrin, which after recrystallizations thrice from ethanol, afforded needles (0.4 g), melting at 196°. $[\alpha]_D^{28}$+84.5° (c, 0.658).

b) Half a gram of β-amyrin acetate of m.p. 230–240° was hydrolyzed in just the same manner as above, to yield β-amyrin, which after recrystallizations thrice from ethanol also afforded needles (0.4 g), melting at 196°. $[\alpha]_D^{28}$+84.3° (c, 0.708).

Anal. Found: C, 84.22; H, 11.95. Calcd. for $C_{30}H_{50}O$: C, 84.44; H, 11.81.

Both of these samples of β-amyrin did not depress the melting point on admixture with an authentic specimen[5c]. The infrared spectra of these samples were also found to be superimposable with that of the authentic specimen of β-amyrin[5c].

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Studies on the Essential Oils of Tobacco Leaves

Part IX. Acid Fraction of the American Flue Cured Tobacco Leaf.

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Constituents of the acid fraction and their contents in the essential oil of the American flue cured tobacco leaf (AFCTL) were determined and compared with those of the Japanese flue cured tobacco leaf (JFCTL)[1]. No difference was found in regard of the kind of volatile organic acid, except that AFCTL contained no α-crotonic acid. On the other hand, the content of the acid fraction in AFCTL was exceedingly higher than that of JFCTL, especially in low molecular fatty acids. Both *n*- and isovaleric acids could not be isolated and identified in AFCTL, as well as in JFCTL, but the presence of β-methylvaleric acid was also proved and it seemed that this acid might be one of the important constituents for the aromatic character of Virginia tobacco leaves.

The American flue cured tobacco leaf (AFCTL) is regarded as a superior material leaf for American type or Straight Virginia type cigarettes, because of its nice smoking quality, and it is also used as the highest material leaf for some high grade cigarettes manufactured in Japan, as well as in other countries. Many tobacco workers have taken some interest in investigating what constituents could cause such excellent smoking aroma or taste, but no reports have yet appeared concerning this problem.

Among the organic acids in the essential oil of tobacco leaves reported in the literature, *n*- and isovaleric acids[2] seemed to be of most interest because of their good aroma. Halle and Pribram[3] had once reported the isolation of isovaleric acid from the essential oil of Hungarian tobacco leaf, but they failed to give any detail in respect of the method of identification in their paper. On the other hand, *n*-valeric acid had been isolated and identified from the essential oil of Algerian tobacco leaf by Sabetay and Panouse[4]. In the previous paper[1], the authors reported that neither *n*- nor isovaleric acid could be isolated in the essential oil of JFCTL. In this paper, the acid fraction in the essential oil of AFCTL was investigated and it was intended to find out whether any difference exists in the kind of volatile organic acid between AFCTL and JFCTL, and in addition to confirm the existence of *n*- and isovaleric acids in AFCTL.

The organic acids which were isolated and identified in the essential oil of AFCTL are similar to those found in JFCTL, except that of α-crotonic acid which is not contained in AFCTL. However, there was a great difference in the amount of organic acids between AFCTL and JFCTL (see Table I.). The AFCTL contains such a large amount of formic acid and acetic acid exceeding 97% of total acid. Especially, formic acid which was negligible in JFCTL, is found in an amount exceeding 20% in AFCTL.

1) I. Ōnishi, and K. Yamasaki, This Bulletin, **19**, 137 (1955).
2) A. Bömer, A. Juckenack und J. Tillamans, *Handbuch der Lebensmittelchem.*, **VI**, 310 (1934).
3) W. Halle and E. Pribram, *Ber.*, **47**, 1394 (1914).
4) S. Sabetay and J. Panouse, *Compt. rend.*, **225**, 887 (1947).

TABLE I
CONTENTS OF VOLATILE ORGANIC ACIDS IN THE ESSENTIAL OIL OF AMERICAN AND JAPANESE FLUE CURED TOBACCO LEAVES

	American Flue Cured Leaf		Japanese Flue Cured Leaf	
	Yield mg/10 kg leaf	% for total acid	Yield mg/10 kg leaf	% for total acid
Formic acid	1240	20.1	trace	—
Acetic acid	4780	77.3	268	49.2
Isobutyric acid	—	—	8	1.5
α-Crotonic acid	—	—	40	7.3
n-Caproic acid	23	0.4	19	3.5
β-Methylvaleric acid	38	0.6	106	19.4
Benzoic acid	27	0.5	34	6.2
Phenylacetic acid	96	1.1	70	12.9
2-Furoic acid+(undetermined)			—	—
Total	6204		545	

EXPERIMENTAL

1) Sample: Fermented flue cured tobacco leaf, grown in the United States, classified as "OAL"[5] (1953 crop) was employed in this study. It is said that "OAL" has a stronger and more excellent aroma and taste than other AFCTL imported from the United States. Therefore, 21.3 kg "OAL" was butted by the usual method as performed in the cigarette manufacturing factories in Japan and cut by a cutting-machine in the usual cutting-width.

2) Preparation of Essential Oils and its Acid Fraction: As described in the previous paper[1], the cut tobacco leaf was steam distilled and the distillate was extracted with ether. From this ethereal solution, the acid fraction (39.9 g) was obtained by the usual method.

3) Separation of Acid Fraction by Column Chromatography: In order to remove the large amount of acetic acid and formic acid, the acid fraction was fractionally distilled under atmospheric pressure and a low-boiling point fraction (20.2 g, b.p. 70–112°) was obtained. The distillating residue was dissolved in ether successively, and extracted with saturated aqueous sodium bicarbonate solution. The sodium bicarbonate solution was acidified with sulfuric acid, saturated with sodium chloride, and extracted with ether. Thus, from this ethereal solution, the high-boiling point fraction (4.55 g) was obtained. Each of the organic acids was separated by the column chromatographic procedures as described in the previous paper. The obtained chromatogram of the low-boiling point fraction is shown in Fig. 1. The high-boiling point fraction (4.55 g) was extracted with hot isooctane two or three times, and the isooctane soluble fraction (802 mg) was separated by column chromatography (Fig. 2).

The isooctane insoluble-fraction was extracted with ether, and the ether soluble-fraction (496 mg) was obtained. It was estimated that this fraction consisted of some resinous acidic substances. A small amount of crystal, which was identified as 2-furoic

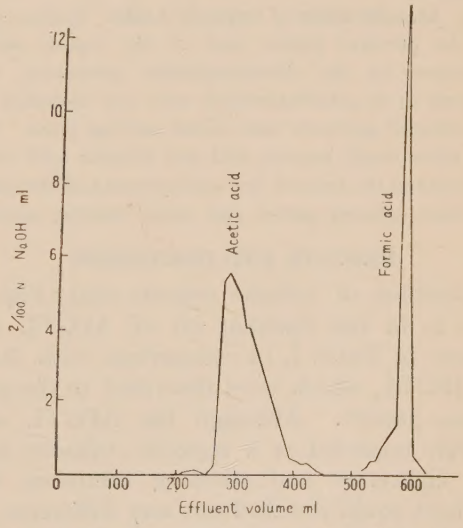

FIG. 1. Chromatographic Separation of low-boiling point Fraction.

Developer used was as follows; Isooctane 450 ml, Isooctane-Ether (10% v/v) 100 ml, Ether 200 ml.
13 g of Silicic acid was used as packing material.

5) "OAL" is an abbreviation of tobacco leaf, used to indicate its growing district, grade and stalk position.

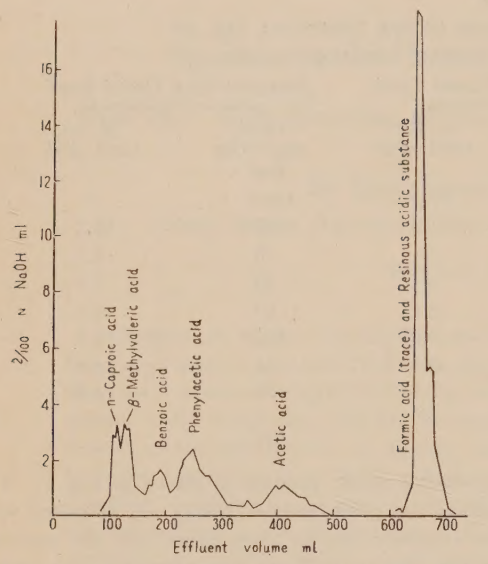

FIG. 2. Chromatographic Separation of high-boiling point Fraction.

Developer used were as follows: Isooctane 600 ml, Isooctane-Ether (10% v/v) 100 ml, Ether 200 ml.

acid, was obtained from the high-boiling point-fraction before that fraction prepared for extraction with isooctane.

4) Identification of Organic Acids: As described in the previous paper, each of the organic acids, separated by the chromatographic procedure, was derived to its *p*-bromphenacyl ester and identified by its infrared spectrum and mixed melting point. On the other hand, benzoic acid and 2-furoic acid were obtained in the form of free acid-crystals and identified by their infrared spectra and mixed melting points.

RESULTS AND DISCUSSION

Contents of volatile organic acid (Figs. 1 and 2) in the essential oil of AFCTL are shown in Table I, in comparison with those of JFCTL, which were described in the previous paper[1]. Although the AFCTL was widely regarded as a superior tobacco leaf for cigarettes and smoking mixtures, the authors could not find out any difference in the kind of volatile organic acids between AFCTL and JFCTL, except that AFCTL contained no α-crotonic acid which was found in JFCTL.

However, with respect to the quantity of organic acid, Table I shows that the amount of total volatile organic acids contained in AFCTL is about ten times larger than that of JFCTL and especially the amount of low-molecular fatty acids (acetic, formic) in AFCTL is twenty times larger than that of JFCTL.

It seemed to be interesting that "OAL" (AFCTL), regarded as a superior flue-cured tobacco leaf, contained such an exceedingly large amount of low-molecular fatty acids, while it is said that these acids would depreciate smoking quality by their strong pungent aroma and sourer taste.

Judging from the fact that AFCTL contains such a large amount of acetic and formic acids, it is considered that AFCTL might be subjected to more intense oxidation than JFCTL, during the process of flue-curing or aging.

Among the volatile organic acids isolated and identified from AFCTL (also-JFCTL), β-methylvaleric acid might be one of the most important acids in relation to the aroma and taste of the flue cured tobacco leaf. This acid had been isolated and identified from the essential oil of Algerian tobacco leaf[4], and from the fatty acid fraction in petroleum[6], but it seems that this acid is not widely found in nature. Neuberg and Rosenberg[7] had reported that a large amount of β-methylvaleric acid appears at the putrefaction of casein by some bacteria, and this acid might be produced by the deamination of isoleucine. It is, however, understood in general, that methyl-ethylacetic acid is produced by the biological degradation of isoleucine through α-keto-β-methylvaleric acid[8]. Recently, Polak[9], in his "Biogenesis of Es-

6) W.A. Quebedeaux, G. Wash, W.C. Ney, W.W. Crench and H.L. Lochte, *J. Am. Chem. Soc.*, **65**, 767 (1943).

7) C. Neuberg and E. Rosenberg, *Biochem. Z.*, **7**, 178 (1908)

8) M.J. Coon and N.S.B. Abrahamsen, *J. Biol. Chem.*, **195**, 805 (1952).

9) E.H. Polak, *Essential Oil Record*, **46**, 369 and 401 (1955); **47**, 6 (1956).

sential Oil," asserted that many constituents having the nonterpenic structure in any essential oil are possibly derived from amino acids and fatty acids, quoting many literature there. According to his opinion, it is possibly understood that β-methylvaleric acid might be derived from isoleucine, but no experimental evidence has yet appeared. Anyway, it is interesting to note that β-methylvaleric acid has surely been isolated and identified only from the tobacco plant.

Acknowledgement. We wish to express our cordial thanks to Prof. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo, for his wise direction and kind guidance, and to Director T. Nakashima, Central Research Institute, Japan Monopoly Corporation, for his strong support. We are also indebted to Miss H. Ōtsuka and Mr. K. Saitō for their cooperation in carrying out this experiment. Last but not least, we are greatly indebted to Emeritus Prof. T. Yabuta for his sound advice.

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 2, p. 86~89, 1957]

Studies on the Essential Oils of Tobacco Leaves

Part X. Acid Fraction in the Essential Oil of the Japanese Flue Cured Tobacco Leaf Before Redrying and Aging.

By Isao Ōnishi and Kyoji Yamasaki

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Received September 19, 1956

Acid fraction of the essential oil of Japanese flue cured tobacco leaf, before redrying and aging (BRA), was compared with that of the same leaf after aging (AA) as reported in previous papers[1,2]. The organic acids which were isolated and identified in the essential oil of BRA are similar to those of AA, except that the former contains propionic and methylethylacetic acids, which were not found in the latter. While, on the other hand, total volatile acids in AA was amounted to about three times as much as that of BRA, and especially the increase of acetic and phenylacetic acids was remarkable during the process of aging.

In the previous papers we have studied the constituents of each fraction in the essential oil obtained from the Japanese flue cured tobacco leaf, after redrying and aging (AA) for in the course of about one year. The same kind of tobacco leaf, having the same grade, stalk position, growing district, before redrying and aging (BRA) was employed in this study in order to investigate changes of the chemical constituents of the essential oil during the process of aging. Besides, we expected to find whether the change of the chemical constituents in the essential oil would suggest the period suitable for the aging of tobacco leaf after it is packed in hogsheads.

From the acid fraction of the essential oil of Japanese flue cured tobacco leaf BRA, the following acids were isolated and identified; formic, acetic, propionic, α-crotonic, methylethylacetic, n-caproic, β-methylvaleric, benzoic and phenylacetic acids.

These organic acids from the essential oil of tobacco leaf BRA are similar to those of AA, except in respect of propionic and methylethylacetic acids which were newly isolated from BRA and acetic and phenylacetic acids contents increased during the process of aging.

However, it was noticed that the amount of acidic resinous constituents, which make the isolation and identification of organic acids (i.e., isooctane insoluble fraction) difficult, in the acid fraction of the essential oil BRA was smaller than that of AA. This fact, which was also observed in the case of the carbonyl fraction[3], would suggest that the resinous substances increased by polymerization of the dynamic substances, so called by Frankenburg[4], during the process of aging.

EXPERIMENTAL

1) Preparation of Essential Oil: The flue cured tobacco leaves, 1955 crop, harvested in Okayama, before redrying and aging, smoking leaf, special grade, were butted and cut by the same method as usually employed in cigarette manufacturing. As previously reported[1], cut tobacco leaf, 135 kg, was steam distilled and the distillate was extracted with ether.

1) I. Ōnishi and K. Yamasaki, This Bulletin, **19**, 137 (1955).
2) I. Ōnishi and K. Yamasaki, This Bulletin, **20**, 68 (1956).
3) I. Ōnishi and M. Nagasawa, This Bulletin, **21**, 43 (1956).
4) W.G. Frankenburg, *Advances in Enzymology*, **10**, 371 (1950).

TABLE I
PHYSICAL AND CHEMICAL CONSTANTS OF THE ESSENTIAL OIL OF JAPANESE FLUE CURED TOBACCO LEAF BEFORE REDRYING AND AFTER AGING

	Before Redrying	After Aging
Yield (%)	0.133	0.215
d_{20}^{20}	0.9840	0.829
n_D^{20}	1.4798	1.4131
Acid value	76.81	—
Saponification value	119.32	—
Ester value	42.51	—

From the ethereal solution, 179.36 g of the essential oil of tobacco leaf BRA was obtained (see Table I.) and the characters of its odor were almost the same as that of the essential oil of tobacco leaf AA.

2) **Preparation of Acid Fraction:** As reported previously, from 179.36 g of the essential oil BRA, 19.05 g of the acid fraction which contained a considerable amount of volatile non-acidic compounds (ether, carbonyl compounds etc.) was obtained. In order to remove the volatile non-acidic compounds and collect the low-boiling point fraction, mainly consisting of acetic acid, the acid fraction was fractionally distilled at atmospheric pressure and 2.066 g of the low-boiling point fraction (b.p. 60–83°, bath temp. 100–170°) was obtained.

The distilling residue was dissolved in ether, and the ethereal solution was extracted with saturated aqueous sodium bicarbonate solution. This aqueous solution was acidified with sulfuric acid, then saturated with sodium chloride and extracted with ether. After removal of ether, 6.918 g of high-boiling point fraction was obtained. This fraction was extracted two or three times with hot isooctane and 2.047 g of the isooctane soluble-fraction was obtained. Table II. shows the yield of acid fraction, isooctane soluble- and insoluble-fraction, in BRA as compared with those of AA.

3) **Separation of Organic Acids:** As reported previously, organic acids were separated by column chromatography employing silicic acid as an adsorbent and isooctane as an eluting solvent. The chromatograms of low- and high-boiling point fractions are represented in Figs. 1 and 2, respectively.

4) **Identification of Organic Acids:** Each organic acid isolated by column chromatography was derived to its *p*-bromphenacyl ester and identified by its melting point, infrared spectrum and elementary analysis.

TABLE II
COMPARISON OF YIELD OF THE ACID FRACTION IN THE ESSENTIAL OIL OF JAPANESE FLUE CURED TOBACCO LEAF BEFORE REDRYING AND AFTER AGING

	Before Redrying	After Aging[a]
Yield of acid fraction (mg/1 kg leaf)	141	474
Acid fraction %/Total essential oil	10.63	16.89
Isooctane soluble (mg/1 kg leaf)	51.4 (36.5%)[b]	125.0 (26.3%)
Isooctane insoluble (mg/1 kg leaf)	15.2 (10.8%)	77.4[c] (16.3%)

a) See previous paper[1].
b) ( ) indicates percentage for the total acid-fraction.
c) The amount of the isooctane insoluble-part of the previous paper is less than that obtained in this study, because the former was refined by extracting the ethereal-solution of the fraction with aqueous sodium bicarbonate.
d) The total sum of the isooctane soluble and insoluble part does not amount to 100%, because the acid fraction contains non-acidic compounds.

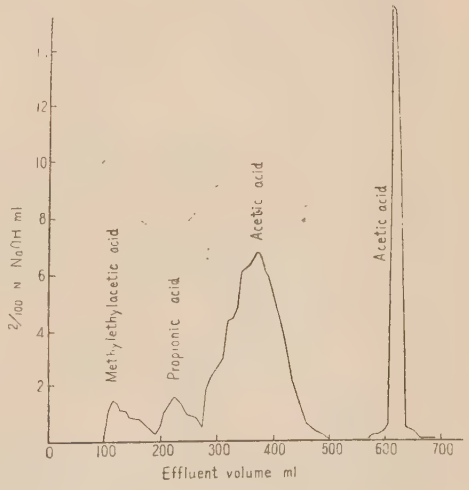

FIG. 1. Chromatographic Separation of Low-boiling Point Fraction.
Developers used were as follows: Isooctane 500 ml, Isooctane-Ether (10% v/v) 100 ml, Ether 200 ml.
13 g. of Silicic acid was used as packing material.

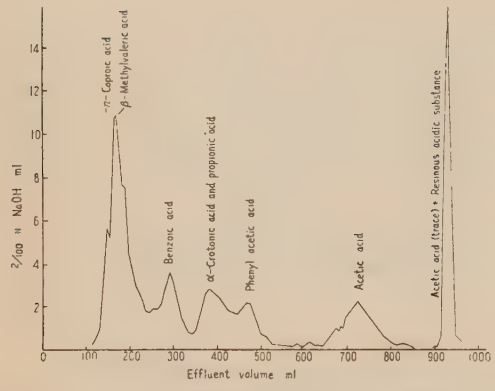

FIG. 2. Chromatographic Separation of High-boiling Point Fraction.

Developers used were as follows: Isooctane 800 ml, Isooctane-Ether (10% v/v) 100 ml, Ether 200 ml.

with each other.

RESULTS AND DISCUSSION

Table III. shows the content of each organic acid isolated and identified from the essential oil before redrying and aging, compared with that of after aging. There was no difference in the kind of organic acid between BRA and AA, except that methylethylacetic and propionic acids were newly isolated from BRA.

However, Table II. shows the increase of total volatile organic acid from 141 mg BRA to 474 mg AA, per 1 kg of tobacco leaf. The content of low-molecular fatty acid, especially acetic acid in the essential oil of tobacco leaf AA was about 2.5 times as much as that of BRA and an increase of phenylacetic acid by 3 times was observed during the process of aging, but the question of the formation of these acids has not been clarified in detail.

TABLE III
COMPARISON OF THE ACID FRACTION IN THE ESSENTIAL OIL OF JAPANESE FLUE CURED TOBACCO LEAF BEFORE REDRYING AND AFTER AGING

	Before Redrying		After Aging	
	mg/10 kg leaf	% for total acid	mg/10 kg leaf	% for total acid
Formic acid	—	—	trace	—
Acetic acid	98	33.9	268	49.2
Propionic acid	9	2.9	—	—
Isobutyric acid	—	—	8	1.5
α-Crotonic acid	22	7.6	40	7.3
Methylethylacetic acid	10	3.5	—	—
n-Caproic acid	15	5.3	19	3.5
β-Methylvaleric acid	82	28.3	106	19.4
Benzoic acid	32	11.2	34	6.2
Phenylacetic acid	21	7.3	70	12.9

5) Identification of Methylethylacetic Acid: *p*-Bromphenacyl ester of the acid corresponding to the first peak in Fig. 1 (m.p. 54–5°), showed no depression of melting point upon admixture with *p*-bromphenacyl ester of authentic methylethylacetic acid (m.p. 55–6°), and infrared spectra of both derivatives agreed well with each other.

Anal. Found: C, 51.88; H, 4.67. Calcd. for $C_{13}H_{15}BrO_3$ (as the methylethylacetic acid derivative): C, 52.19; H, 5.05.

6) Identification of Propionic Acid: *p*-Bromphenacyl ester of the acid corresponding to the second peak in Fig. 1 (m.p. 58–60°) showed no depression of melting point, upon admixture with *p*-bromphenacyl ester of authentic propionic acid (m.p. 58–9°) and infrared spectra of both derivatives agreed completely

Phenylacetic acid is distributed in many plants, for example, in Japanese peppermint oil, being found as an ester with β,γ-hexenol[5]. In the process of biological degradation of phenylalanine by microorganism[6,7], phenyl-

5) N. Hirao, "Nihon-Seiyu-Kagaku", Sasaki-tosho Pub. Co., Tokyo, 1939, p. 99.

6) K. Felix, K. Zorn und H. Dirr-Kaltenbach, *Z. physiol. Chem.*, **247**, 141 (1937).

7) T. Uemura, *J. Agr. Chem. Soc. Japan*, **13**, 1146 (1937).

acetic acid is produced through phenylpyruvic acid and Polak[8] reports, that this process would be observed in the biogenesis of phenylacetic acid in plants. Although phenylalanine has been found in the tobacco leaf after flue-curing[9], it is not experimentally proved whether phenylacetic acid is produced from the phenylalanine contained in the tobacco leaf.

α-Crotonic acid was not generally found in plants except of its presence in *Ramalina reticulata*[10] and animal fat[11], but it is considered that this acid might be produced from the fatty acid-cycle of Lynen[12], which plays an important role in the biogenesis of fatty acids.

8) E.H. Polak, *Perfum. Ess. Oil Record*, **46**, 369, 401 (1955); **47**, 6 (1956).
9) S. Ranjan and M.M. Laloraya, *Nature*, **177**, 235 (1956).
10) J.B.Stark, E.D. Walter and H.S.Owens, *J. Am. Chem. Soc.*, **72**, 1819 (1950).
11) F.L. Brensch and E. Ulysoy, *Arch. Biochem.*, **11**, 489 (1946).
12) F. Lynen, *Angew. Chem.*, **67**, 463 (1955).

Acknowledgement. We wish to express our cordial thanks to Prof. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo, for his wise direction and kind guidance, and also to Director T. Nakashima, Central Research Institute, Japan Monopoly Corporation, for his strong support to us. We are indebted to Dr. M. Matsui, Department of Agricultural Chemistry, University of Tokyo, for the microanalysis and Miss H. Ōtsuka, Mr. K. Saito for their cooperation in carrying out this experiment. Thank are also due to Mr. J. Kobata, Takamatsu Local Monopoly Bureau, for his cordial cooperation in sampling of tobacco leaf. Last but not least, we are greatly indebted to Emeritus Prof. T. Yabuta for his sound advice.

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Studies on the Essential Oils of Tobacco Leaves

Part XI. Phenol Fraction (3)

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The previously estimated carbonyl compound[1] contained in the caustic alkali soluble-fraction of the essential oil of the Japanese flue cured tobacco leaf was identified as crotonaldehyde, and the presence of α-pyrrylmethylketone in this phenol fraction was also proved. On the other hand, the constituents and contents of carbonyl and phenolic compounds, contained in this fraction before redrying, were compared with those of after redrying and aging. In the course of redrying and aging stages, phenolic compounds having the carbonyl group and the carbonyl compounds of this fraction increased, but the phenolic compounds having no carbonyl group decreased. Methylsalicylate could not be found in that fraction before redrying, but *m*-cresol was newly isolated and identified.

The carbonyl compound previously estimated from the caustic alkali soluble-fraction (CASF) of the Japanese flue cured tobacco leaf (JFCTL) after redrying and aging was identified as crotonaldehyde by the mixed melting point and infrared spectrum of its 2,4-dinitrophenylhydrazone (2,4-DNPHone) with the authentic compound. Whereas, the other compound which was estimated as α-pyrrylmethylketone by its infrared spectrum was proved to be identical with the authentic compound by subsequent investigation.

On the other hand, from the CASF contained in the essential oil of JFCTL prior to redrying, α-pyrrylmethylketone, salicylaldehyde, *o*-hydroxyacetophenone, benzaldehyde and acetaldehyde were isolated and identified as their 2,4-DNPHone by the application of mixed melting point and infrared spectra procedures.

The caustic alkali soluble-fraction, after removal of the carbonyl compounds, was separated into two fractions, i.e., the phenol-fraction (1) and the high molecular fatty acid-fraction (2). The phenolic compounds were derived into their azo-compounds coupled with diazotized sulfanilic acid and from these derivatives phenol, guaiacol, eugenol and *m*-cresol were identified by their developed colors and R_F values on the paper chromatograms. Whereas, on the other hand, the phenolic compounds were derived into their azocompounds coupled with diazotized *p*-nitroaniline, and separated by columnchromatography, employing alumina as an adsorbent and monochlorbenzene as an eluting solvent. After each band was refined, dissolved in caustic isopropyl alcohol, their contents were determined by their optical densities in the absorption spectrum.

On the other hand, the high molecular fatty acid-fraction was investigated by the reverse-phase paper chromatography as reported by Inoue and Noda[2]. Thus, the presence of palmitic, myristic and lauric acid was proved. Among these acids, palmitic acid was proved to be the main component of this fraction.

No remarkable difference was to be found among the kind of the phenolic compounds

1) I. Ōnishi and K. Yamamoto, This Bulletin, **20**, 70 (1956).

2) Y. Inoue and M. Noda, This Bulletin, **19**, 214 (1955).

in the essential oil of JFCTL before redrying and after aging. However, in the essential oils of before redrying, methylsalicylate was not found and *m*-cresol was newly isolated and identified.

Although the carbonyl compounds of the essential oil decrease in the course of redrying and aging, it is interesting to note that the phenolic compounds having the carbonyl group increase during the same stages.

Rayburn[3] had isolated *m*-cresol in tobacco smoke, but no report can be found in concern of the presence of *m*-cresol and crotonaldehyde in the essential oil of tobacco leaves.

EXPERIMENTAL

I) Identification of Crotonaldehyde: A yellow precipitate, obtained from Band No. 2 of Table II. reported in the previous paper[1], was proved as a mass of small needle crystal under the microscope. The mixed melting point of this 2,4-DNPHone, m.p. 190°, with the authentic crotonaldehyde derivative, m.p. 192°, showed no depression and both infrared spectra agreed well with each other.

II) Identification of α-Pyrrylmethylketone: The previously estimated band, as α-pyrrylmethylketone was repeatedly collected by the same procedure employing a column chromatographic separation. Dark black-purple crystals were obtained by repeated recrystallization from pyridine containing a small amount of *n*-hexane and the melting point of this crystal was raised to 298° with decomposition which was identical with that of the authentic derivative.

III) Isolation and Identification of Carbonyl Compounds in the Phenol Fraction: As described in the previous paper[1], the ethereal-solution of CASF, isolated from 134 kg of JFCTL before redrying, was shaken with saturated sodium bisulfite solution and 2,4-DNPHone of the carbonyl compounds, 112 mg, was obtained. The 2,4-DNPHone was separated by a liquid chromatography, employing a mixture of silicic acid and Celite as an adsorbent and a mixture of petroleum ether (b.p. 40–80°) and ether as an eluting solvent. Therefore, six bands were separated. Benzaldehyde, acetaldehyde, *o*-hydroxyacetophenone, salicylaldehyde and α-pyrrylmethylketone were isolated in their regular eluting order. Each band was identified by its infrared spectrum and mixed melting point with the authentic derivative.

An unknown carbonyl 2,4-DNPHone, m.p. 134°, reported in Band No. 1 of Table II. in the previous paper[1] was also isolated, but it was not identified. Further investigation is now in progress. The constituents and contents of the carbonyl compounds contained in the phenol-fraction of the essential oil of JFCTL before redrying and after aging, were compared and results are summarized in Table I.

IV) Fractionation of Phenolic Compounds and High Molecular Fatty Acids: After removal of car-

3) C.H. Rayburn, W.R. Harlan and H.R. Hiumer, *Anal. Chem.*, **25**, 1419 (1953).

Table I

Constituents and Contents of Carbonyl Compounds Contained in the Phenol Fraction of Essential oil of JFCTL Before Redrying and After Aging

	Before Redrying		After Aging	
	Isolated 2,4-DNPHone from 134 kg leaf	Free Carbonyl mg/100 kg leaf	Isolated 2,4-DNPHone from 120 kg leaf	Free Carbonyl mg/100 kg leaf
α-Pyrrylmethylketone	45	12.0	115	34.1
Acetaldehyde	21	3.1	42	6.9
Salicylaldehyde	14	4.3	28	9.5
o-Hydroxyacetophenone	10	3.2	12	4.3
Benzaldehyde	2	0.5	5	1.5
m-Tolualdehyde	—	—	4	1.3
Crotonaldehyde	—	—	2	0.5
p-Anisaldehyde	—	—	1	0.3
Unknown	2	0.4	3	0.8
Total	94	23.5	212	59.2

Contents of these carbonyl compounds contained in the essential oil of JFCTL after aging, were estimated from the results shown in Tables I and II in the previous paper.

bonyl compounds, the ethereal-solution of the phenolic fraction was decolorized with active carbon and filled up to just 1000 ml with ether. In order to fractionate phenolic compounds and high molecular fatty acids, two procedures were tried.

1) Steam distillation method—An alliquot, 100 ml, of the ethereal-solution, containing crude phenolic compounds, was poured into a distillating flask and the solvent removed so as to retain a small amount of ether. The residual liquid was conducted by a steam distillation until the distillate showed no phenolic color-reaction by caustic alkali solution and diazosulfanylic reagent and the distillating speed was controlled so as to ensure that no high molecular fatty acids might be distilled out. Both the distillate and distillating residue were saturated with sodium chloride, each extracted with ether, then both ethereal-solutions were dried with anhydrous sodium sulfate and these solutes were determined.

2) Fractional precipitation method—Another alliquot, 100 ml, was poured into an Erlenmeyer flask, the ether distilled off, and after 70 ml of 95% methyl alcohol was added, it was heated on a water bath for one minute. After the dissolved solution was left in an ice box for twenty-four hours, a white crystal consisting of high molecular fatty acids separated. The filtrated crystal was washed with cold methyl alcohol and weighed after drying. From the filtrate, the solvent was removed under depressed pressure, passing nitrogen gas and the residue weighed. The determined results of these two fractions are shown in Table II.

TABLE II
COMPARISON OF STEAM DISTILLATING AND FRACTIONAL PRECIPITATING METHODS FOR THE SEPARATION OF PHENOLS AND HIGH MOLECULAR FATTY ACIDS

	Phenols (g)	High Molecular Fatty Acids (g)
1) Steam distillating method	0.1955 0.2112	1.6369 1.6420
2) Fractional precipitating method	0.2482 0.2504	1.5732 1.5592

The fractional precipitating method seems not to be so complete for the separation of both components, because a small amount of the high molecular fatty acids remains in the filtrate and some loss of the phenols was unavoidable in the distillation procedure under depressed pressure during the removal of solvent from the filtrate. Consequently, the steam distillating method seems to be preferable than the fractional precipitating method.

The light red colored non-volatile fraction was solidified at room temperature, and after standing for a few days almost all of the fraction turned into a white crystal which had a slight acidic odor.

The light yellow colored volatile fraction, non-viscous fluid, had a strong odor resembling cresol. This fraction was derived into azo-compound and separated by paper chromatography, in the usual manner. The developed R_F values and colors of spots were as follows; 0.37-pink, 0.22-yellow, 0.13-yellow and 0.08-orange. Among these four spots, three spots except 0.22-yellow were identified as eugenol, phenol and guaiacol when compared with those after redrying and aging, as reported in the previous paper[1].

V) Isolation and Content Determination of Phenols: The phenol-fraction which was obtained by the above mentioned steam distillating procedure, was conducted with further separation according to Stoltenberg[4] and Rayburn's[5] method. The phenolic-fraction was dissolved in 5 ml of aqueous solution of N-sodium hydroxide and diluted to 100 ml with distilled water. An alliquot, 12.5 ml, of this solution was again diluted to 100 ml, and 12.5 ml of aqueous solution of 2 N-sodium hydroxide was added. The solution obtained was cooled in an ice box under 5° and 12.5 ml of 0.1 N-diazo-reagent[5] was added. After standing for a few minutes, it was acidified to congo-red with 2.5 ml of 20% sulfuric acid and extracted with ether. The ethereal-solution was washed with water, dried with anhydrous sodium sulfate and the solvent removed. The residual precipitate was dissolved in 100 ml of monochlorbenzene and from this solution, 50 ml was used for column chromatographic separation. The "Brockman" alumina used as an adsorbent was weakened of its adsorpting activity by the addition of 4% distilled water, so as to control the separation to an optimum condition and development was conducted under atmospheric pressure, employing monochlorbenzene as an eluting solvent. Six bands were separated on the column. In order

4) H. Stoltenberg, *Z. anal. Chem.*, **146**, 181 (1955).

5) 0.1 N-diazo-reagent was prepared as follows: *p*-nitroaniline, 6.9 g, was dissolved in a mixture of hydrochloric acid, 32 ml (d = 1.16), and 100 ml of water on a water-bath, then it was cooled under 5°. An aqueous solution of N-sodium nitrite, 50 ml, which was cooled under 5°, was added and filled up to 500 ml with ice water. The mixture obtained must be stored in an ice box. It was filtered prior to use.

Table III
Contents of Phenols in the Essential Oil of JFCTL Before Redrying and After Aging

Band No.	Wave length, measured (mμ)	Compound	Contents (mg/100 kg leaf)	
			Before Redrying	After Aging
1	530	—	—	—
2	390	Eugenol	298.5	25.8
3	545	Guaiacol	333.6	37.6
4	560	*m*-Cresol	144.0	none
5	510	Phenol	620.8	41.1
6	505	—	—	—
Total			1396.9	104.5

to make the volume uniform, the solvent was removed from the eluates under depressed pressure. An alliquot of each eluate was refined by a successive column chromatographic procedure, employing alumina which contains no water as an adsorbent and isopropyl alcohol which contains 1% water as an eluting solvent. Before the bottom band had just eluted out, the eluting solvent was removed by a compressed nitrogen gas and the major band cut off. The separated band was extracted with an aqueous solution of 75% isopropyl alcohol, containing 1% of an aqueous solution of N-sodium hydroxide, and the extracted solution filled up to 100 ml. On the other hand, standard solutions were arranged, each of them containing 1 mg of each phenolic compound in 100 ml of the above mentioned isopropyl alcohol. The absorption spectra of the eluates and standard solutions were compared by use of the Beckman spectrophotometer and the contents of phenols were determined. The results are shown in Table III. According to Helmuth and Rayburn, Band No. 1 which is composed of a mixture of some kind of phenols, appeared as a light colored broad band consisting of some different colors, and Band No. 6 is a mixture of diazo-compounds which were produced as by-products in the preparation of those azo-compounds. As the amount of these by-products was so small and did not move in the eluting procedure it was considered that it might be negligible.

Band No. 4: This band corresponds to the yellow spot of R_F value 0.22 in the paper chromatography mentioned above and the wave length of the maximum absorption in spectrum, 560 mμ, was identical with that of authentic *m*-cresol.

VI) Identification of High Molecular Fatty Acids in the Phenol Fraction: The steam distillating residue in the above mentioned V. of this study seemed to be composed of a mixture of some kinds of high molecular fatty acids. Therefore, it was derived into 2,4-dinitrophenylhydrazid and identified by a reverse phase chromatography[2]. 2,4-Dinitrophenylhydrazids of the high molecular fatty acids were prepared by a simple method, and developed by a mixture of 90% (v/v) methylalcohol-acetic acid-tetralin (10 : 2 : 1 by volume) on the Tōyō filter paper No. 50. Consequently, palmitic, myristic and lauric acids were identified.

RESULTS AND DISCUSSION

The carbonyl compounds which remained unknown in the previous paper were identified as crotonaldehyde and α-pyrrylmethylketone. On the other hand, the phenol-fraction of the essential oil of JFCTL before redrying was investigated. From the carbonyl fraction contained in the phenol-fraction α-pyrrylmethylketone, salicylaldehyde, *o*-hydroxyacetophenone, acetaldehyde and benzaldehyde were isolated and identified and their contents compared with those after redrying and aging. The content of carbonyl compounds in this fraction from before redrying is smaller than that of after redrying and aging. Consequently, it seemed that they increased during the redrying and aging stages. Especially, the carbonyl compounds, having the phenolic hydroxyl group in their molecules, increased during the same stages. However, the carbonyl compounds which isolated from the carbonyl fraction as their sodium bisulfite compounds, decreased during the same stages[6]. On the other hand, the phenolic

6) I. Ōnishi and M. Nagasawa, This Bulletin, **21**, 43 (1957).

compounds having no carbonyl group surely decreased durig the same stages. It is interesting to discuss on these differences as changes of their contents seemed to be caused by some physiological reasons in the course of fermentation or aging of tobacco leaf but no conclusive details could be obtained.

The fact that the carbonyl compounds having no phenolic hydroxyl group such as acetaldehyde, benzaldehyde and α-pyrrylmethylketone were identified in this CASF, may be attributed to their water soluble-qualities, especially, for in this study many solvents were used for fractionation.

As for crotonaldehyde, we have no report at hand in concern of its presence in nature, so it is presumed to be one of the products decomposed by steam distillation.

According to Naghski et al.[7], the content of phenol in the American fire cured tobacco leaf decreases to about one-ninth during the aging stage, and this is assumed to be caused by the natural oxidation resulting polymerized substances. In the case of our JFCTL, it was found that the change of content of phenolic compounds in the aging stage was similar to that of the American fire cured tobacco leaf.

Acknowledgement. We wish to express our cordial thanks to Prof. Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo, for his wise direction and kind guidance, and to Director T. Nakashima, Central Research Institute, Japan Monopoly Corporation, for his strong support. We are also indebted to Mr. H. Takahara and Mr. K. Saitō for their cooperation in carrying out this experiment. Last but not least, we are greatly indebted to Emeritus Prof. T. Yabuta for his sound advice.

7) J. Naghski, E.G. Beinhart and J.F. Couch, *Ind. Eng. Chem.*, **36**, 556 (1944).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 2, p. 95~98, 1957]

Studies on the Essential Oils of Tobacco Leaves

Part XII. Carbonyl Fraction (4)

By Isao Ōnishi and Masatoshi Nagasawa

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The constituents and their contents of carbonyl compounds contained in the essential oil of Japanese Burley Tobacco Leaf (JBTL) were investigated and compared with those of Japanese Flue Cured tobacco leaf (JFCTL). Benzaldehyde, acetaldehyde, furfural, 5-hydroxymethyl-furfural, isobutyraldehyde and C_6-aldehyde were isolated from the carbonyl-fraction in the essential oil of JBTL. A remarkable characteristic of the essential oil of JBTL is that benzaldehyde was proved as a predominant constituent and the content of furfural was exceedingly smaller than that of JFCTL. 5-Methylfurfural, found in the essential oil of JFCTL, could not be isolated from that of JBTL.

In the previous paper[1], the constituents and their contents of the essential oil of Japanese Flue Cured tobacco leaf (JFCTL) before redrying were compared with those of after aging. The present study was undertaken in order to investigate the constituents and their contents of the essential oil of Japanese Burley tobacco leaf (JBTL) and to compare them with those of JFCTL. It was expected that there would be remarkable differences of the constituents between the essential oil of JBTL and those of JFCTL, because the chemical constituents and pH values of both leaves are quite different, as stated: JBTL contains a very small amount of sugar, a large amount of volatile bases and its pH value was more basic as compared with JFCTL[2]. Furthermore, the kinds and contents of amino acids[3] of JBTL differ from those of JFCTL.

In this study, the essential oil of JBTL after aging was prepared by steam distillation and separated into basic, acidic, phenolic, carbonyl and neutral fractions, as previously described in Part I[4].

From the carbonyl fraction, the following carbonyl compounds were isolated and identified: benzaldehyde, acetaldehyde, furfural, 5-hydroxymethylfurfural, isobutyraldehyde and C_6-aldehyde. 5-Methylfulfural and acetone, which were found in JFCTL, could not be isolated in this study.

All the isolated 2,4-dinitrophenylhydrazones (2,4-DNPHones) were identified by their melting points and infrared spectra, as described in the previous paper[5].

EXPERIMENTAL

(I) Separation and Identification of Carbonyl Compounds:

Employing the steam distillating procedure as described in Part I of this study[6], the essential oil was obtained from 124 kg of JBTL ("Mito No. 3 Strain", 1st grade smoking leaf, 1955 crop). The 2,4-DNPHones of the carbonyl compounds were prepared by a method similar to that described in Part VII of this study[5]. Consequently, a low boiling point carbonyl-fraction (I), 4.230 g, and a high boiling point carbonyl-fraction (II), 12.320 g, were obtained.

1) I. Ōnishi and M. Nagasawa, This Bulletin, **21**, 43 (1957).
2) J. Kobata, et al., *Sci. Papers Cent. Res. Inst. Japan Monop. Corp.*, **96**, 40 (1956).
3) M. Nagasawa, and K. Yamamoto, *Sci. Papers Cent. Res. Inst. Japan Monop. Corp.*, **96**, 50 (1956).
4) I. Ōnishi, and K. Yamasaki, This Bulletin, **19**, 137 (1955).
5) I. Ōnishi, and M. Nagasawa, This Bulletin, **19**, 143 (1955).
6) I. Ōnishi, and M. Nagasawa, This Bulletin, **21**, 38 (1957).

Table I
Chromatographic Separation of 2,4-DNPHones Isolated from the Low Boiling Point Carbonyl-Fraction in Essential Oil of Burley Tobacco Leaf

Band No.	Yield (mg)	Compound (2,4-DNPHone)	Color
5	1.3	Unknown	Red
4	102.5	Acetaldehyde	Yellow
3	5.5	Benzaldehyde	Red-yellow
2	4.6	Isobutyraldehyde	Yellow
1	2.0	C_6-aldehyde	Yellow

Band number indicate order of elution.
Adsorbent: Silicic acid+Celite (2:1 by weight).
Column: 200×36 mm.
Developing solvent: 1–6% Ether in Petroleum benzine (b.p. 40–80°).

Table II
Chromatographic Separation of 2,4-DNPHones Isolated from the High Boiling Point Carbonyl-Fraction in Essential Oil of Burley Tobacco Leaf

Band No.	Yield (mg)	Compound (2,4-DNPHone)	Color
7	20.0	Unknown	Red
6	12.0	Furfural	Red
5	5.5	5-Hydroxymethylfurfural	Dark red
	4.1	Benzaldehyde	Red-yellow
4	39.6	Benzaldehyde	Red-yellow
3			
2	3.5	Unknown	Red-yellow
1			

Footnotes are the same as those of Table I.

1) Chromatographic Separation of 2,4-DNPHones from the Low Boiling Point-Carbonyl Fraction (I): The 2,4-DNPHones (I), 120 mg, were separated by the method, described in Part II[5]. The results obtained are shown in Table I.

Isolation of C_6*-aldehyde:* Band No. 1, recrystallized from ethanol, afforded yellow needles, m.p. 127°. The infrared spectrum agreed completely with that of the C_6-aldehyde derivative, reported in the previous paper[6].

Identification of benzaldehyde: Band No. 3, recrystallized from ethanol-ethylacetate, afforded red-yellow crystals, m.p. 241°. The infrared spectrum agreed completely with that of the authentic benzadehyde derivative. Although Band No. 3 was developed by petroleum benzine-benzene, as described in Part II[5], no crystals of 5-methylfurfural derivative could be obtained.

2) Chromatographic Separation of 2, 4-DNPHones from the High Boiling Point Carbonyl-Fraction (II): The 2,4-DNPHones (II), 90 mg, were separated by the method, as described in Part II[5]. The results obtained are shown in Table II.

Bands No. 1, 2 and 3: Each band afforded red oils, so that they could not be identified.

Identification of benzaldehyde: Band No. 4, recrystallized from ethanol-ethylacetate, afforded red-yellow crystals, m.p. 243°. The infrared spectrum agreed completely with that of the authentic benzaldehyde derivative. Although Band No. 4 was developed by petroleum benzine-benzene as described in Part II[5], no crystals of 5-methylfurfural derivative could be isolated.

Identification of benzaldehyde and 5-hydroxymethylfurfural: Band No. 5, recrystallized from ethanol-ethylacetate, afforded dark red crystals. However, these were separated into two bands by a successive rechromatography. The bottom band, recrystallized from ethanol-ethylacetate, afforded red-yellow crystals, m.p. 240°. The infrared spectrum agreed completely with that of the authentic benzaldehyde derivative. The top band, recrystallized from ethanol-ethylacetate, afforded dark red crystals, m.p. 191°. The infrared spectrum agreed well with that of the authentic 5-hydroxymethylfurfural derivative[6].

Identification of furfural: Band No. 6. recrystallized from ethanol-ethylacetate, afforded red crystals. The infrared spectrum agreed well with that of the mixture of *cis*- and *trans*-furfural derivatives[5].

TABLE III
CONTENTS OF CARBONYL COMPOUNDS FOUND IN THE CARBONYL FRACTION OF THE ESSENTIAL OILS OF BURLEY AND FLUE CURED TOBACCO LEAVES

Constituents	Burley		Flue Cured	
	%	mg/kg of leaf	%	mg/kg of leaf
Benzaldehyde	46.6	18.5	7.6	1.8
Acetaldehyde	14.4	5.7	5.3	1.3
Unknown	19.6	7.8	11.1	2.6
Furfural	11.6	4.6	50.4	11.8
5-Hydroxymethylfurfural	6.3	2.5	7.5	1.8
Isobutyraldehyde	1.0	0.4	2.0	0.5
C_6-aldehyde	0.5	0.2	1.0	0.3
5-Methylfurfural	—	—	14.6	3.4
Acetone	—	—	0.5	0.1
Total	100.0	32.7	100.0	23.6

Bands No. 7 and 8: These bands merely afforded red colored oils, by even any further development, so that their identification was impossible.

RESULTS AND DISCUSSION

A comparison of the constituents and their contents of carbonyl compounds in the essential oils of JFCTL[1] and JBTL is shown in Table III.

It is remarkable characteristic of the carbonyl-fraction of JBTL that benzaldehyde was found to be a predominant constituent, corresponding to about a half-portion of the total carbonyl-fraction which was ten times higher than that of JFCTL, and moreover, the content of furfural did not exceed a half of that of JFCTL. 5-Methylfurfural could not be isolated.

Generally, benzaldehyde is contained in plants in the form of glycosides[7),8)]. These glycosides are hydrolyzed by the enzymes and weak acids producing *d*-glucose, hydrogencyanide and benzaldehyde. However, no report has yet been made on the presence of such glycosides in tobacco leaves. On the other hand, the presence of hydrogencyanide[9)] and benzaldeyde[10)] has already been reported in tobacco smoke; yet, it is certain that most of the hydrogencyanide might be derived by heat decomposition in smoking from many compounds which contain nitrogen. While, no report is available in concern of the presence of both compounds in tobacco leaves, except on the point that benzaldehyde has been isolated and identified in the essential oil of JFCTL, by the authors.

The contents of benzaldehyde in the essential oil of the JFCTL obtained by the steam distillation and the ether-extraction are similar to each other[11)]. Therefore, benzaldehyde in JBTL would be transferred into the essential oil without undergoing any change by the steam distillating procedure.

It is generally considered that 5-hydroxymethylfurfural is derived from hexose, and furfural is derived from pentose, pentosan and uronic acids in plants. Consequently, it is assumed that the amount of 5-hydroxymethylfurfural in the essential oil of JBTL might be smaller than that of JFCTL, because contents of the soluble-sugar of JBTL, most of the sugar consisting of hexose, are so small amounting to about only 0.3%[2)], corresponding to about one-sixtyth of that of JFCTL.

7) G. Klein, "Handbuch der Pflanzenanalyse", II Band, Spezielle Analyse Erster Teil p. 271 and 291, (1932).

8) K. Paech, and M.V. Tracey, "Modern Methods of Plant Analysis", Vol. II, p. 434 and Vol. IV, p. 676, (1955).

9) E.L. Wynder, "Biologic Effects of Tobacco", Little Brown and Company, Boston, p. 21, (1955).

10) C. Neuberg, and J. Burkard, *Biochem. Z.* **243**, 472 (1931).

11) I. Onishi, and M. Nagasawa, **20**, (1956).

However, the results obtained indicate that the essential oil of JBTL contains more 5-hydroxymethylfurfural than that of JFCTL. Although the contents of pentose, pentosan and uronic acids in JBTL and JBCTL are not studied yet, the above results might have be attributed mainly to the fact that the pH value of JBTL is more basic.

It is also interesting to note that 5-methylfurfural could not be isolated, in this study. This seems to have been caused by the fact that JBTL contains a very small amount of methylpentose.

The contents of isobutyraldehyde and C_6-aldehyde in the essential oil of JBTL are similar to those of JFCTL.

Acknowledgement. We wish to express our cordial thanks to Professor Y. Sumiki, Department of Agricultural Chemistry, University of Tokyo, for his wise direction and kind guidance, and to Director T. Nakashima, Central Research Institute, Japan Monopoly Corporation, for his strong support given. We are also indebted to Miss K. Yamamoto and Mr. K. Saitō for their cooperation in carrying out this experiment. Last but not least, we are greatly indebted to Emeritus Professor T. Yabuta for his sound advice.

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Studies on the Protease of *Pseudomonas*

Part III. Specific Action of the Protease

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Received September 29, 1956

The specific action of the crystalline protease obtained from *Ps. myxogenes* sp., was examined from its action on various proteins, determination of the N-terminal residues of the enzymatically degraded gelatin — which was most readily digested by the enzyme — and comparison with other proteinases, such as trypsin, papain and pepsin. It was shown that the enzyme might be classified as a collagenase and the mode of digestion of gelatin had a more close resemblance to papain than trypsin or pepsin.

INTRODUCTION

Gelatinolytic enzymes (collagenases) appear to have extremely wide spread distribution in the world of bacteria[1]. However, as it was pointed out by Yamamoto[2], specificity of the proteinases originating from different bacterial species seems to differ among each other. For example, it has been shown by Yonezawa[3] that a conversion of crystalline egg-albumin to plak-albumin is not observed by treatment with the crystalline protease* of *Ps. myxogenes* sp., but it is possible by the enzyme of *B. subtilis*[4].

In order to examine the specificity of the crystalline protease obtained from *Ps. myxogenes* sp., digestion of some proteins (gelatin, hide-powder, gluten, haemoglobin, casein and egg-albumin) was studied in comparison with other known proteinases such as trypsin, papain and pepsin. Moreover, further determination was made on the N-terminal residues of the enzymatically degraded gelatin using Sanger's fluorodinitrobenzene technique[5].

1) Emil L. Smith, "The Enzymes" Vol. 1, p. 862 (1951).

2) T. Yamamoto, "Biseibutsu Kōgyo" editted by Asai (Asakura Shoten) p. 522 (1956).

3) D. Yonezawa, Private communication.

* Supplied by the author.

4) N. Egg-Larsen, K. Linderstrøm-Lang, and M. Ottesen, *Arch. Biochem.*, **19**, 340 (1948).

EXPERIMENTAL AND METHODS

Materials: The twice recrystallized protease[6] of *Ps. myxogenes* sp. (abbrev. *Ps.* enzyme), crystalline trypsin (Mochida & Co., Ltd.), purified pepsin (Wako & Co., Ltd., 1 : 10000), and crude papain (Merck & Co., Ltd., 1 : 350) were used for comparative study.

Proteolytic Degradation: An appropriate amount of enzyme was added to 60 cc (or 20 cc) of a 5% (or 7.5%) substrate solution adjusted to the pH-optima of each protease — i.e., *Ps.* enzyme at pH 7.0, trypsin at 8.0, papain at 6.0 with Sörensen's phosphate buffer, and pepsin at pH 2.2 with Kolthoff's citrate buffer — placed in a 100-cc Erlenmeyer flask previously kept at 35° for 30 minutes, and the mixture brought to reaction at 35° in addition with 1 cc of toluene. Only in the case of papain, HCN was supplemented in a concentration of 0.3% to the substrate solution for activation.

Determination of Proteolysis: Non protein nitrogen (N.P.N) was determined by adding 3 cc of digest to 6 cc of 30% trichloroacetic acid at room temperature and this was kept for 30 minutes and filtered. A semimicro-Kjeldahl nitrogen determination was performed on the filtrate. Amino nitrogen (NH_2-N) was determined on 5 cc of the digest according to the method of Pope & Stevens[7].

N-Terminal Determination: The method used

5) F. Sanger, *Biochem. J.* **39**, 507 (1945).

6) K. Morihara, This Bulletin. **21**, 11 (1957).

7) C.G. Pope & M.F. Stevens, *Biochem. J.* **33**, 1070 (1939).

here was the same as that of A. Courts[8,9,10].

To 20 cc of degraded gelatin solution (7.5%), 6 N-HCl (or 8% $NaHCO_3$) was added to adjust the pH to 1.5 (to 7.0 with pepsin) to inactivate proteolytic activity. After more than two hours' storage at 30°, the solution was neutralized with 8% $NaHCO_3$ and made up to 25 cc with water. A mixture composed of 5 cc of the digest, 5 cc of water, and 10 cc of 8% $NaHCO_3$ to adjust the pH to 8.5, was shaken mechanically with 0.6 cc of FDNB for 24 hrs. The solution was initially kept at 35°, then brought to room temperature after an elapse of about one hour.

Supplementation of 5 g of $MgSO_4$ and acidification with N-HCl caused a yellow gel (DNP-derivatives of the hydrolysate) to be deposited. If the degradation is mild, the mother liquors contain only a negligible quantity of soluble-DNP-peptides (presumably large molecules) which may be neglected, as was shown by A. Courts[10]. But the greater the degradation, more of the DNP-peptides (presumably small molecules) may become soluble in the mother liquor by the treatment. Thus, the experimental value on the high degradation may become smaller than the exact value. The yellow gel thus obtained was dried on P_2O_5 and NaOH, crushed, and washed with acetone until no further yellow colour was removed. The powder was dried at 105° and allowed to equilibrate with the atmosphere.

Fifty mg of the powder was heated with 5 cc of 6 N HCl for 16 hrs. in a sealed-tube at 100°. This causes a destruction of free DNP-proline and DNP-hydroxyproline, while the unchanged amounts of DNP-alanine, DNP-glycine, DNP-glutamic acid, DNP-serine and DNP-aspartic acid were 80%, 40%, 75%, 85% and 60% respectively. After cooling, the hydrolysates (mixtures composed of DNP-amino acid) were extracted three times with an equal volume of ether.

The ether soluble-DNP-amino acid mixtures thus obtained were separated chromatographically on buffered Celite* columns. DNP-aspartic acid (R 0.10), DNP-serine (R 0.15) and DNP-glutamic acid (R 0.4) were separated using a column of 4.5 g Celite (0.5 cc stationary phase/1 g Celite) buffered at pH 4 with phosphate-citric buffer (54 cc 0.2 M Na_2HPO_4+46 cc 0.2 M citric acid) and chloroform-ether (90 : 10 by vol.) as the organic phase eluent, and DNP-glycine (R 0.46) using chloroform as the solvent. By a column of 3 g Celite buffered at pH 6 (77.5 cc Na_2HPO_4+22.5 cc citric acid) using chloroform, DNP-alanine (R 0.15) was separated. These elutions may be speeded up by the addition of ether.

Each DNP-amino acid solution collected was taken to dryness, dissolved in 10 cc of 1% $NaHCO_3$ and estimated colorimetrically with the use of the Beckmann photometer, at 360 mμ. The quantity of N-terminal residues (moles) released was calculated from optical density and the destructive factor from the hypothesis that DNP-derivatives used for acid-hydrolysis was 100,000 g (approximate to the moles of gelatin), and the ash content and moisture of each sample were the same.

8) A. Courts, *Biochem. J.* **58**, 70 (1954).
9) A. Courts, *Biochem. J.* **58**, 74 (1954).
10) A. Courts, *Biochem. J.* **59**, 382 (1955).
* Celite 545, Johns Manville Co.

RESULTS

1) Optimum pH and Temperature. The course of action of *Ps.* enzyme on gelatin at various pH values and temperatures was measured as shown in Figs. 1 and 2. The optimum pH was found to be in the range 7.0–8.5, the optimum temperature was approximately 45°.

2) Rates of Hydrolysis of Substrates. The action of *Ps* enzyme on various proteins was studied and the results are shown in Fig. 3. As it can be seen, this enzyme attacks gelatin and hide-powder greatly but the other proteins slightly.

From the course of digestion of the sub-

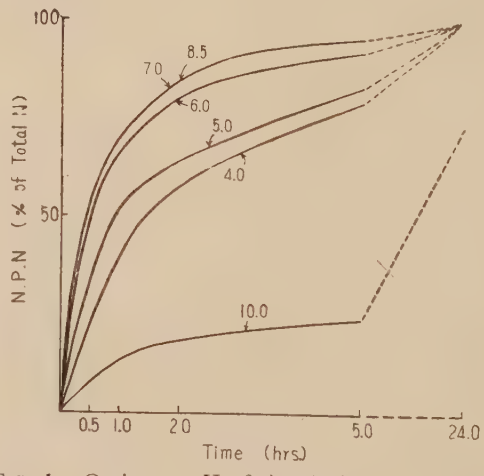

FIG. 1. Optimum pH of the Action of the Crystalline Protease.

Concentration of substrate was 5% and 1 mg/60 cc of the enzyme was used.

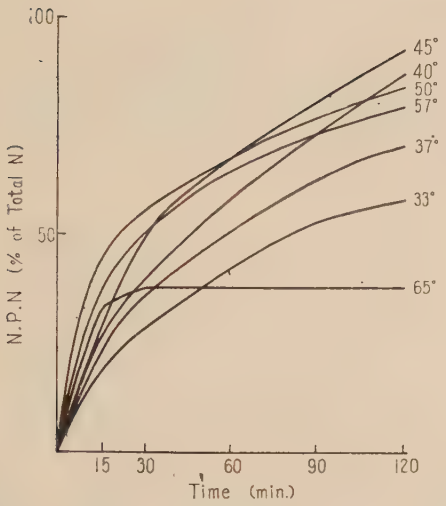

FIG. 2. Optimum Temperature of the Action of the Crystalline Protease.

Concentration of substrate was 5%, and 0.5 mg/60 cc of enzyme was used.

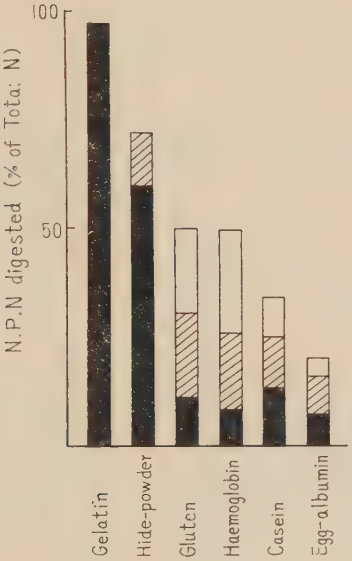

FIG. 3. Comparison of the Rates of Hydrolysis of Various Substrates by the Crystalline Enzyme.

Concentration of substrate was 5%, and 1 mg/60 cc of enzyme was used. The reaction was carried out at 35° for 5 hrs. (▬), 24 hrs. (▨) and 48 hrs. (□).

strates, they were divided into 3 groups, i.e. 1) gelatin and hide powder, 2) gluten and haemoglobin, and 3) casein and egg-albumin. The digestion of Group 1 was rapid in the early stages, up to the formation of 100% N.P.N by digestion of gelatin and 60% N.P.N by hide powder. But the extend of digestion of hide powder on over 60% N.P.N was considerably slow. The initial digestion of Group 2. seemed to be very slow, but kinetics of the reaction almost obeyed the second-order reaction ($K=1/t \cdot x/a(a-x)$). That is, when the substrate concentration was 5% and 1 mg/60 cc enzyme was used in the reaction at 35°, $K \times 10^4$ was calculated as 2.05 in gluten and 1.7 in haemoglobin. Moreover,

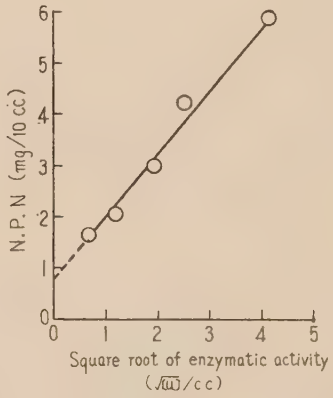

FIG. 4. Activity of the Enzyme on Gluten.

Substrate was 1% and contained Sörensen's phosphate buffer of pH 7.0. The reaction was carried out at 35° for 24 hrs.

activity curves[11] were found to be represented by the formula: $x=Kt\sqrt{E}$, where x = percentage digestion; K = constant; t = time; E = percentage of enzyme concentration, as is shown in Fig. 4. The digestion of Group 3. in comparison with the former two groups described was very slow in all stages.

The splitting action on peptides or acylated amino acids such as glycylglycine, diglycylglycine, glycyl-L-leucine, glycyl-DL-leucine, glycyl-DL-phenylalanine, glycine-ethyl ester and acetyl-DL-methionine was not observed by examination of amino acid liberated with the use of a paper-partition chromatography.

Next, experiments were made to determine

11) E. Bidwell and W.E. Heyningen, *Biochem. J.* **42**, 140 (1948)

the difference of action with the other known proteinases on various substrates. The course of action of each enzyme on gelatin with the requisite amount of enzyme (mg/60 cc) is shown in Fig. 5. From the determination of the N.P.N ((A) in Fig. 5), it is found that

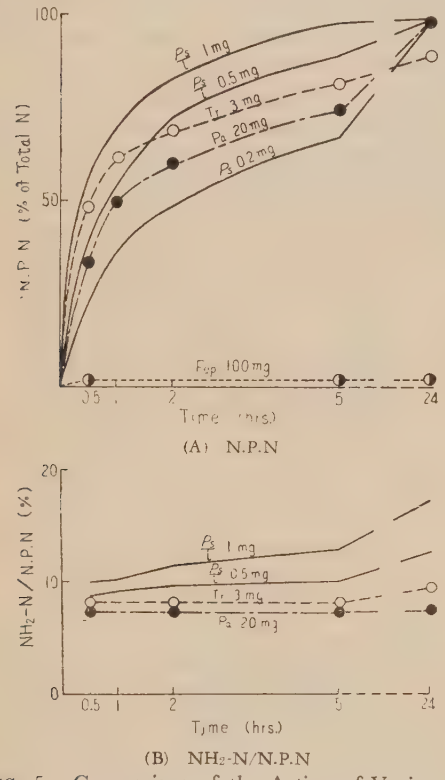

FIG. 5. Comparison of the Action of Various Proteases on Gelatin.

Requisite amounts of enzyme were added as was shown in the figure to 60 cc of 5% substrate solution and the reaction was carried out at 35°.

——— crystalline protease of *Ps. myxogenes* sp. (*Ps*)
○----○ crystalline trypsin (Tr)
◑ · · · ◑ purified pepsin (Pep)
●— —● crude papain (Pa)

Ps enzyme acts remarkably well on gelatin in comparison with the others, the digestion rate per mg of *Ps* enzyme being about 5 to 10 times stronger* than that of trypsin, and about 40–80 times that of crude papain. Pepsin hardly digests gelatin under these conditions. Interestingly, the action of trypsin is rapid in the early stages up to the formation of 60% N.P.N after which the extend of digestion slows down in comparison with the *Ps* enzyme (this reason will be discussed later on).

The relationship between the liberated N.P.N and NH_2-N ((B) in Fig. 5) is shown to be linear except with the *Ps* enzyme, which shows formation of more NH_2-N per N.P.N with the elapse of time and increase of the enzyme content suggesting that it acts still more on the gelatin-hydrolysate not precipitated with trichloroacetic acid up to the formation of about 17% NH_2-N (per N.P.N). The value of NH_2-N per N.P.N is about 8–17% (varying with time or the enzyme content) in *Ps* enzyme, 7.5–9.5% in trypsin, and 7.5% in papain. This means that *Ps* enzyme attacks more bonds with peptide linkage of gelatin in comparison with trypsin or papain. The next experiment supports this view more clearly. After the addition of appropriate amounts of *Ps* enzyme to the digest of trypsin (90% N.P.N liberated, and NH_2-N/N.P.N was 9.5%) the mixture was kept for one night at 35°. The formation of NH_2-N increased greatly and reached 17% (per N.P.N). On the other hand, even with the addition of trypsin to the digest of *Ps* enzyme, not any increase of the NH_2-N was observed. Fukumoto, et al.[12] using preparations of crystalline proteinase from *B. amyloliquefacans* Fukumoto have reported that NH_2-N formation by the action on gelatin or casein was less than that of trypsin. Perhaps, the *Ps* enzyme may be a different type of proteinases.

Liqueficating activity (from N.P.N) of *Ps* enzyme on the other substrates was compared with those of trypsin, papain and pepsin, and the results are shown in Table I.

Subsequent to the activity of each proteinase

* The results measured by the viscosity method ((u)/mg), as shown in Part II of this series and the results obtained by determination of N.P.N liberated in this experiment are approximately the same as those in the reaction for 30 minutes.

12) J. Fukumoto & H. Negi, *Symposia on Enzyme Chemistry* (Japan), 7, 12 (1952).

TABLE I

COMPARISON OF THE ACTIVITY OF *Ps* ENZYME ON SUBSTRATES WITH THE OTHER PROTEINASES

The values shown in the Table show the activity ratio of each enzyme when compared with *Ps* enzyme, calculated from enzyme amounts necessiated for the same degree of digestion (N.P.N%) as that of *Ps* enzyme. Substrate concentration was 5%, and the reaction was carried out at 35° for 5 hrs.

Substrate	Rate of activity			
	Ps enzyme*1	trypsin*1	papain*2	pepsin*3
Gelatin	1	0.15	0.02	0.00
Hide-powder	〃	0.15	0.02	0.00
Gluten	〃	0.4	1.5	0.05
Haemoglobin	〃	0.5	0.2	0.1
Casein	〃	5	0.9	0.04
Egg-albumin	〃	10	3	0.7

*1 crystalline material
〃2 crude 〃 (Merck, 1 : 350)
〃3 purified 〃 (Wako, 1 : 10000)

on the substrates being calculated as standard with the activity of *Ps* enzyme (as 1), substrate specificity of each enzyme was found to be differentiable with each other. From the comparative study, it is shown that *Ps* enzyme digests gelatin and hide-powder remarkably well, trypsin digests egg-albumin and casein, and papain (even crude material) digests strongly egg-albumin, gluten and casein. Consequently, the author considers that *Ps* enzyme may be classified as a collagenase.

Further determination revealed that the NH_2-N formation (per N.P.N) of *Ps* enzyme and the extend of digestion of *Ps* enzyme with the time elapse were generally higher than those of the other proteinases (i.e., *Ps* enzyme > trypsin > papain).

3) N-Terminal Residue of Enzymatically Degraded Gelatin. In order to examine the specific action of *Ps* enzyme more clearly, the N-terminal residues of the enzymatically degraded gelatin was determined by Sanger's fluorodinitrobenzene technique. This technique does not permit the bonds broken to be specified, but its application indicates that the amino acid residues carrying the amino groups are released during hydrolysis.

Digesting the gelatin with *Ps* enzyme, the N-terminal residues were measured with the time elapse (Table II), and the enzyme exhibited a marked specificity for peptide bonds involving the amino group of alanine in the initial stage of reaction (within 30 minutes). However, with reaction exceeding five hours, the specificity was shown to be scattered in all bonds of amino groups without distinction. That is to say, primary specificity is shown to be alanine at the site of amino groups of the peptides, and secondary specificity is observed in rapid success in all the other amino groups.

The change in end groups at various temperatures in the digestion of gelatin with *Ps* enzyme is summarized in Table III. Thermal degradation under these conditions was negligible. The lower the temperature, the less N-terminal residues were released, and the residue liberated primarily (i.e., as shown at the low temperature), is clearly shown to be alanine similary as in the initial stage of reaction in the former experiment. At high temperatures, the specificity was scattered too widely, and not distinguished.

Effect of the pH on degradation was measured and the results are shown in Table IV. The primary specificity is shown to be alanine by the degradation at pH 4.0 and 9.0–10.5, at which the reaction was mild. At pH 5.5–7.0 the degradation was remarkably increased and so the specificity was not distinguished. At pH 9.0–10.5, only alanine groups increase remarkably, while little increase of the other

TABLE II

LIBERATION OF N-TERMINAL RESIDUES DURING THE DEGRADATION OF GELATIN BY *Ps* ENZYME

Into 20 cc of a 7.5% solution of gelatin containing Sörensen's phosphate buffer of pH 7.0, 0.8 mg of *Ps* enzyme was added and the mixture was allowed to react at 35° for a suitable time in addition with 0.5cc of toluene.
End groups are expressed as moles/100,000 g DNP-gelatin (or-hydrolysate).

Time of reaction (min.) / Amino-acid (M)	0	10	30	60	120	300	2400
Aspartic acid+Serine	0.09	0.60	1.04	1.82	2.23	3.55	5.21
Glutamic acid	0.12	3.33	4.00	4.42	5.20	5.75	8.03
Glycine	0.39	1.98	2.26	3.58	4.22	5.00	14.91
Alanine	0.08	4.83	6.44	7.08	7.20	6.95	7.79
Approx. increase in alanine		×60	×80	×86			
Approx. increase in glutamic acid		×28	×33	×37			

* The value of the control was almost equal to that of zero hour's reaction, and therefore negligible.

TABLE III

EFFECT OF TEMPERATURE ON THE DEGRADATION OF GELATIN BY *Ps* ENZYME

For conditions, see Table II. The reaction was carried out for 1 hr. at various temperatures as shown in the Table. End groups are expressed as moles/100,000 g DNP gelatin (or-hydrolysate).

Temperature (C°) / Amino-acid (M)	15	25	35	45	55
Aspartic acid+Serine	0.70	1.41	1.82	2.73	2.40
Glutamic acid	1.47	3.57	4.42	5.86	5.08
Glycine	2.52	3.02	3.58	4.74	4.58
Alanine	5.40	6.73	7.08	7.16	6.70
Approx. increase in alanine	×67	×84	×86		
Approx. increase in glutamic acid	×12	×30	×37		

TABLE IV

EFFECT OF pH VALUES ON THE DEGRADATION OF GELATIN BY *Ps* ENZYME

For conditions, see Table II. Various buffer solutions were prepared. Michaelis acetate buffer at pH 4.0, Sörensen's phosphate buffer at pH 5.5–7.0, Clark & Lub's borate buffer at pH 9.0, and Kolthoff's buffer at pH 10.5–12. The reaction was carried out at 35° for 2 hours. End groups are expressed as moles/100,000 g DNP-gelatin (or-hydrolysate).

pH / Amino-acid (M)	4.0	5.5	7.0	9.0	10.5	12
Aspartic acid+Serine	0.74	3.41	2.23	0.87	0.94	0.11
Glutamic acid	1.67	5.81	5.20	1.44	1.07	0.08
Glycine	1.46	4.41	4.22	6.26	4.80	0.52
Alanine	3.21	5.60	7.20	8.37	8.24	0.16
Approx. increase in alanine	×40	×70	×90	×105	×103	
Approx. increase in glutamic acid	×14	×45	×43	×12	×9	
Approx. increase in glycine	×3.7	×11.3	×10.8	×16	×12	

TABLE V

EFFECT OF ENZYME CONCENTRATION ON THE DEGRADATION OF GELATIN BY *Ps* ENZYME

Various amounts of *Ps* enzyme were used for the degradation, other conditions being the same as in Table II. The reaction was carried out at 35° for 5 hrs. End groups are expressed as moles/100,000 g DNP-gelatin (or-hydrolysate).

Amino-acid (M) \ Amounts of enzyme added (mg/20 cc)	0.00	0.01	0.03	0.1	0.3	0.8
Aspartic acid+Serine	0.09	0.66	0.74	1.05	3.60	3.55
Glutamic acid	0.12	1.77	1.82	2.40	4.74	5.75
Glycine	0.39	2.15	1.74	2.35	4.24	5.00
Alanine	0.08	4.07	3.42	4.13	5.60	6.95

residues occur which differentiates this from the degradation at pH 5.5–7.0.

Effect of enzyme concentrations on the degradation of gelatin was measured and the results are shown in Table V. When a low concentration of enzyme was used, the primary specificity on alanine appeared as the N-terminal residue released, while when a high concentration of enzyme was used all the other residues also increased without distinction.

Obvious results on the degradation of gelatin under various conditions show that *Ps* enzyme initiately attacks bonds involving alanine groups as N-terminal residues, and in rapid succesion splits bonds containing other amino groups as N-residues. The reason for the excellent extend of the degradation of *Ps* enzyme may be attributable to the extensive specificity on gelatin.

The digestions of gelatin were determined by examining the difference of specificity between *Ps* enzyme and other known proteinases (Table VI).

From Table VI, it is shown that trypsin attacks the peptide-bonds involving the amino group of glycine, and papain on bonds involving alanine. The rate of hydrolysis of gelatin by pepsin was comparatively slow under these conditions, and the residues liberated were not obscure. The above results of the digestion by trypsin and papain are the same as those obtained by A. Courts[10].

The mode of attack of the four proteinases on gelatin are compared in Table VII.

As shown in Table VII, the specificity of

TABLE VI

N-TERMINAL RESIDUES RELEASED IN GELATIN BY TRYPSIN, PAPAIN AND PEPSIN

Appropriate contents of each enzyme were added into 20 cc of 7.5% gelatin solution containing Sörensen's phosphate buffer of pH 8.0 in the case of trypsin, pH 6.0 in the case of papain (containing 0.3% HCN for activation), and Kolthoff's citrate buffer of pH 2.2 in the case of pepsin. The mixed solution was kept at 35°. End groups are expressed as moles/100,000 g DNP-gelatin (or-hydrolysate).

Amino-acid (M) \ Enzyme used	trypsin 3 mg		crude papain 20 mg		purified pepsin 50 mg	
	2.5 hrs.	25 hrs.	2.5 hrs.	25 hrs.	5 hrs.	40 hrs.
Aspartic acid+Serine	0.81	1.30	1.51	2.15	0.38	1.11
Glutamic acid	1.08	1.56	2.72	4.84	0.54	1.23
Glycine	8.00	10.63	7.05	9.03	0.63	2.46
Alanine	0.88	1.84	5.21	6.36	0.75	2.17
Approx. increase in glycine	×20.5		×18			
Approx. increase in alanine			×57			

TABLE VII
N-TERMINAL RESIDUES RELEASED DURING THE PROTEOLYSIS OF GELATIN

Proteinase	Primary specificity	Secondary specificity
Ps enzyme	alanine	glutamic acid, glycine, aspartic acid+serine (very intense)*
trypsin	glycine	glutamic acid, alanine, aspartic acid+serine (mild)*
papain	alanine	glutamic acid, glycine, aspartic acid+serine (intense)*
pepsin	—	alanine, glycine, glutamic acid, aspartic acid+serine (very mild)*

* Degree of liberation in secondary specificity.

Ps enzyme on gelatin observed from N-terminal residues liberated resembles to that of papain, but not trypsin and pepsin. It may be considered that the low extend of digestion of gelatin, over 60% N.P.N by trypsin in comparison with *Ps* enzyme, is attributed to its weak splitting of the secondary specificity.

DISCUSSION

From observations on the character of the crystalline protease of *Ps. myxogenes* sp. in comparison with the well known proteinases such as trypsin, papain, and pepsin from some view points, it can be summarized as follows: (1) *Ps* enzyme is not affected by HCN, and the pH optima is 7.0–8.5. The results show that *Ps* enzyme seems to be similar to trypsin and not to papain and pepsin. (2) From the substrate specificity, *Ps* enzyme seems to be a collagenase clearly differentiable from trypsin, papain and pepsin. (3) The N-terminal residues released from the enzymatic-hydrolysate of gelatin, show that *Ps* enzyme is similar to papain and not to trypsin and pepsin.

From these three points, it may be concluded that *Ps* enzyme is a collagenase not resembling to trypsin, papain and pepsin, but is closer to papain than trypsin and pepsin from the mode of action.

SUMMARY

1) Experiments were conducted on the enzymatic action of the crystalline protease obtained from cultures of *Ps myxogenes* sp., and results obtained are as given below (2)–(4).

2) The optimum pH for liquefaction of gelatin was found to be 7.0–8.5, and the optimum temperature about 45°.

3) The enzyme acted remarkably on gelatin and hide-powder, but slightly on the other proteins, i.e., gluten, haemoglobin, casein, egg-albumin. The substrate-specificity is clearly differentiable from those of trypsin, papain and pepsin. Consequently, the enzyme may be assumed to be a collagenase.

4) From the mode of digestion of gelatin when the N-terminal residue technique was applied, it can be said that *Ps* enzyme is more similar to papain, than trypsin and pepsin.

The author wishes to express his sincere thanks to Prof. H. Katagiri of Kyoto Univ. for his constant guidance and encouragement in the course of this work, and also to Mr. E. Masuo of this Laboratory.

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Studies on the Essential Oil of *Mentha rotundifolia*

Part II. Structure of Rotundifolone, a New Terpenic Ketone.*

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Received October 4, 1956

Rotundifolone $C_{10}H_{14}O_2$, the principal component of the essential oil obtained from Japanese *Mentha rotundifolia*, has been found to be 1-methyl-4-isopropylidene-1,2-epoxy-cyclohexanone-3, which was reduced and rearranged to diosphenol. It is a noteworthy fact that the new ketone seems to be one of the intermediates between carvone series and menthone series.

In the previous paper[1] the present author isolated a new α,β-unsaturated ketone $C_{10}H_{14}O_2$ (m.p. 27.5°, λmax 260 mμ) and named it rotundifolone (I). This compound shows a negative ferric chloride reaction and reduces both Fehling's and ammoniacal silver solution. Its Malaprade reaction is positive after being allowed to stand for several hours.

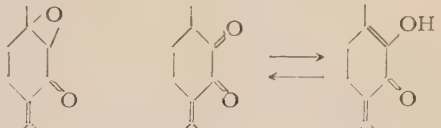
The oil (II)[1], regenerated from rotundifolone semicarbazone consumed 0.7 molar equivalent of hydrogen over palladium-barium sulfate. From the hydrogenated oil, a crystal melting at 82° was separated and established to be identical with diosphenol (III), by the mixed melting point[2].

In the catalytic hydrogenation using a palladium catalyst, rotundifolone absorbed 1 molar equivalent hydrogen to yield dihydrorotundifolone (IV), which was laevorotatory and had a menthone-like odor. The hydrogenated oil showed a negative ferric chloride reaction and easily gave monosemicarbazone of m.p. 210° (dec.), which had two absorption maxima each at 224 mμ and 243 mμ. Analysis of the substance indicated the empirical formula to be $C_{11}H_{19}O_2N_3$, corresponding to the calculated value for dihydrorotundifolone semicarbazone.

Consequently, it was found that rotundifolone (I) and dihydrorotundifolone (IV) were unstable in acid media and readily changed into enolic substances; namely 1-methyl-4-isopropylidene-cyclohexen-1-ol-2-one-3 (II′) was produced from (I), and diosphenol (III) from (IV).

By treating rotundifolone (I) with 5% sulfuric acid and with perbenzoic acid, isorotundifolone $C_{10}H_{14}O_2$ (m.p. 79°) and epoxide (VI) $C_{10}H_{14}O_3$ (m.p. 78°) were obtained respectively. This epoxide (VI), in a cold dilute sulfuric acid solution, was easily hydrolysed to the diol (VII) $C_{10}H_{16}O$ (m.p. 136°), from which acetone was yielded by periodic acid oxidation. The infrared spectra of (I), (VI) and (VII) are shown in Fig. 1.

From these experimental results, the structure of rotundifolone was proved to be either (I) or (I′a).



(I) (I′a) (I′b)

It has been established that 1,2-cyclohexadione exists as monoenol in many cases except

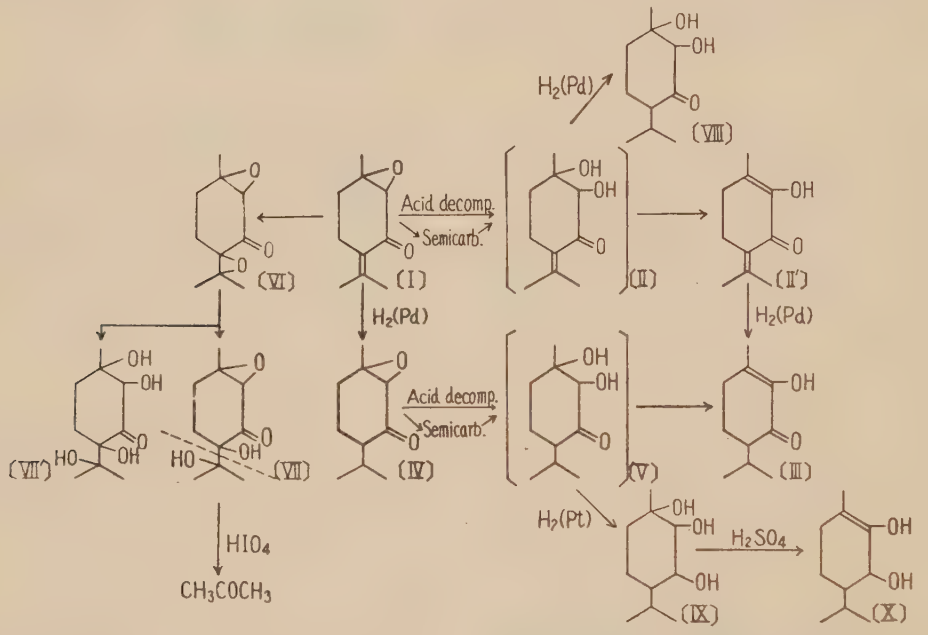
* This report was presented at the Annual Meeting of Agric. Chem. Soc. Japan., Oct. 29 (1955) at the Univ. of Shizuoka, and on March 30, (1956) at the Univ. of Tokyo.

Part I. This Bulletin, **20**, 84 (1956).

1) S. Shimizu, This Bulletin, **20**, 84 (1956).

2) Authentic diosphenol was prepared from *l*-menthone, according to the method reported by Asahina and Mitsuhori in *J. Pharm. Soc. Japan*, **482**, 2 (1922).

when α-carbons are completely alkylsubstituted[3]. The infrared spectrum of neither rotundifolone nor its epoxide showed the existence of a hydroxyl group, moreover these substances show a negative ferric chloride reaction. Consequently, the structure of rotundifolone was not diketoform (I'a) but 1-methyl-4-isopropylidene-1,2-epoxy-cyclohexanone-3 (I). The formation of diosphenol and other substances from (I), is shown in the following scheme.



The hydrogenation of the alkali insoluble-part (V), of the oil regenerated from (IV)-semicarbazone by using a platinum catalyst gave long needles melting at 95°. It showed a typical Malaprade reaction and gave a negative ferric chloride reaction. From the result of analysis, this substance was confirmed to be a triol $C_{10}H_{20}O_3$ (IX), supported by intense absorption of hydroxyl groups in its infrared spectrum (Fig. 2). The triol (IX) was proved to have a tertiary hydroxyl group from the fact that it gave an enolic substance, by treatment with dilute sulfuric acid.

As a hydrogenation product of (II), the compound $C_{10}H_{18}O_3$ (m.p. 85°) was obtained and it was related to (VIII).

When rotundifolone was reduced with lithium aluminium hydride, the reaction mixture was decomposed into a crystalline diol, $C_{10}H_{18}O_2$, of m.p. 113° with dilute sulfuric acid, whereas two diols, $C_{10}H_{18}O_2$, of m.p. 85° and m.p. 145°, respectively were obtained with water. The fact that the diol of m.p. 113° consumed two moles of periodic acid[4] indicated the presence of α-glycol grouping, as shown in formula (XI) which was also supported by its infrared spectrum (Fig. 2). While on the other hand, formulas (XII) and (XIII) were assigned to the diols of m.p. 85° and m.p. 145°, respectively after consideration of results of the periodic acid test. The infrared spectra of these diols are shown in Fig. 2.

In catalytic hydrogenation with palladium, the diol (XII) consumed one molar hydrogen

3) N.J. Leonard and P.M. Mader, *J. Am. Chem. Soc.*, **72**, 5383 (1950).

4) Periodic acid has been proved to be a reagent applicable in the detection of exocyclic double bond besides α-glycol. A. Chatterjee and S.G. Majumdar, *Anal. Chem.*, **28**, 878 (1956).

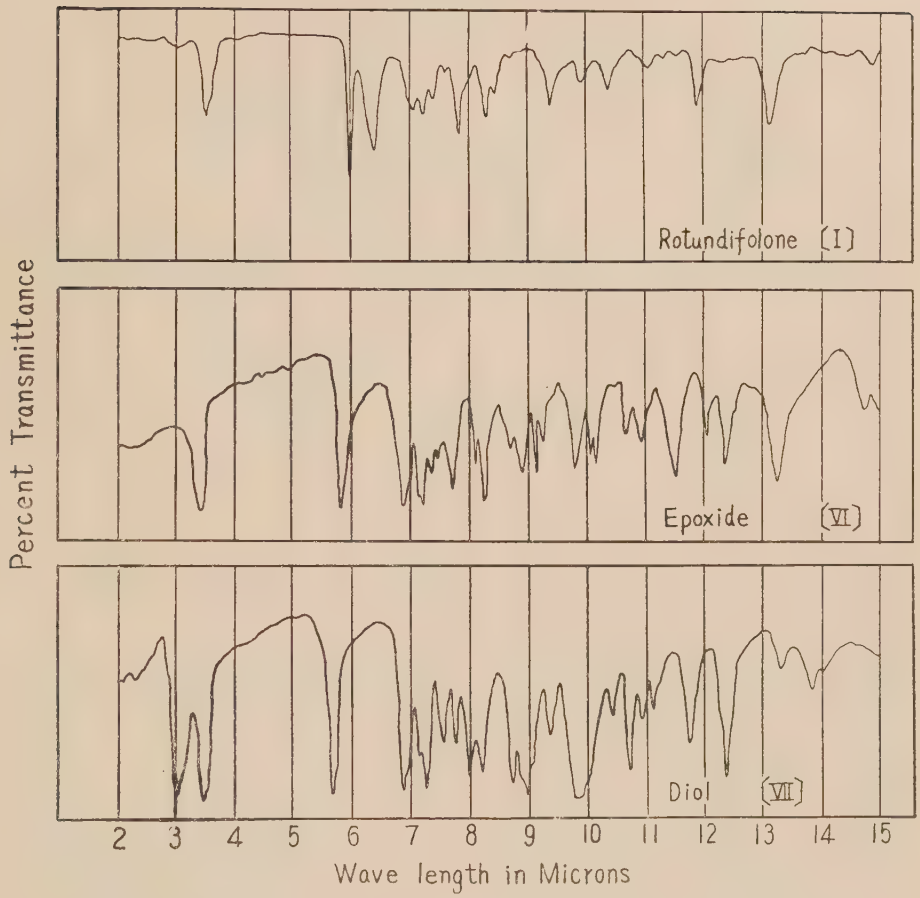


FIG. 1. Infrared Absorption Spectra of Rotundifolone and its Derivatives.

to yield a syrupy dihydro-diol (XVI), and the diol (XIII) also gave a dihydro-diol (XIV), which was found to be identical with one of the lithium aluminium hydride reduction products of (IV).

In this reduction, a syrupy diol $C_{10}H_{20}O_2$ (XV), which showed a positive Malaprade reaction, was obtained from (IV) as the main product. The configuration of these diols will be reported later.

Rotundifolone which has two oxygen atoms on both 2- and 3- carbon of the *p*-menthane ring, is considered as an interesting substance from the biochemical point of view. Cytogenetical studies show that *Mentha rotundifolia* has the least number of chromosomes and is supposed to be one of the prototypes or types connected to the prototypes of *Mentha*[5]. This interpretation seems to support the view that rotundifolone will be a "turn-table" between the menthone and the carvone series, in place of diosphenol*.

In 1955, K.K. Chakravarti et al.[6] isolated a new ketone $C_{10}H_{14}O_2$ from Indian *M. viridis* and named it "mintglyoxal". The infrared spectra of their

5) N. Ikeda and S. Udo, *Scient. Rep. Fac. of Agric., Okayama Univ.* **8**, 62 (1956).

* J. Walker and J. Read stated that diosphenol may be used as a "turn-table" between the menthone and carvomenthone series (*J. Chem. Soc.* 239 (1934)). But diosphenol, a component of the leaves of *Barosma*, has not been isolated as a natural product from the essential oil of the genius *Mentha*.

6) K.K. Chakravarti and S.C. Bhattacharyya, *P.E. O.R.* 256 (1955).

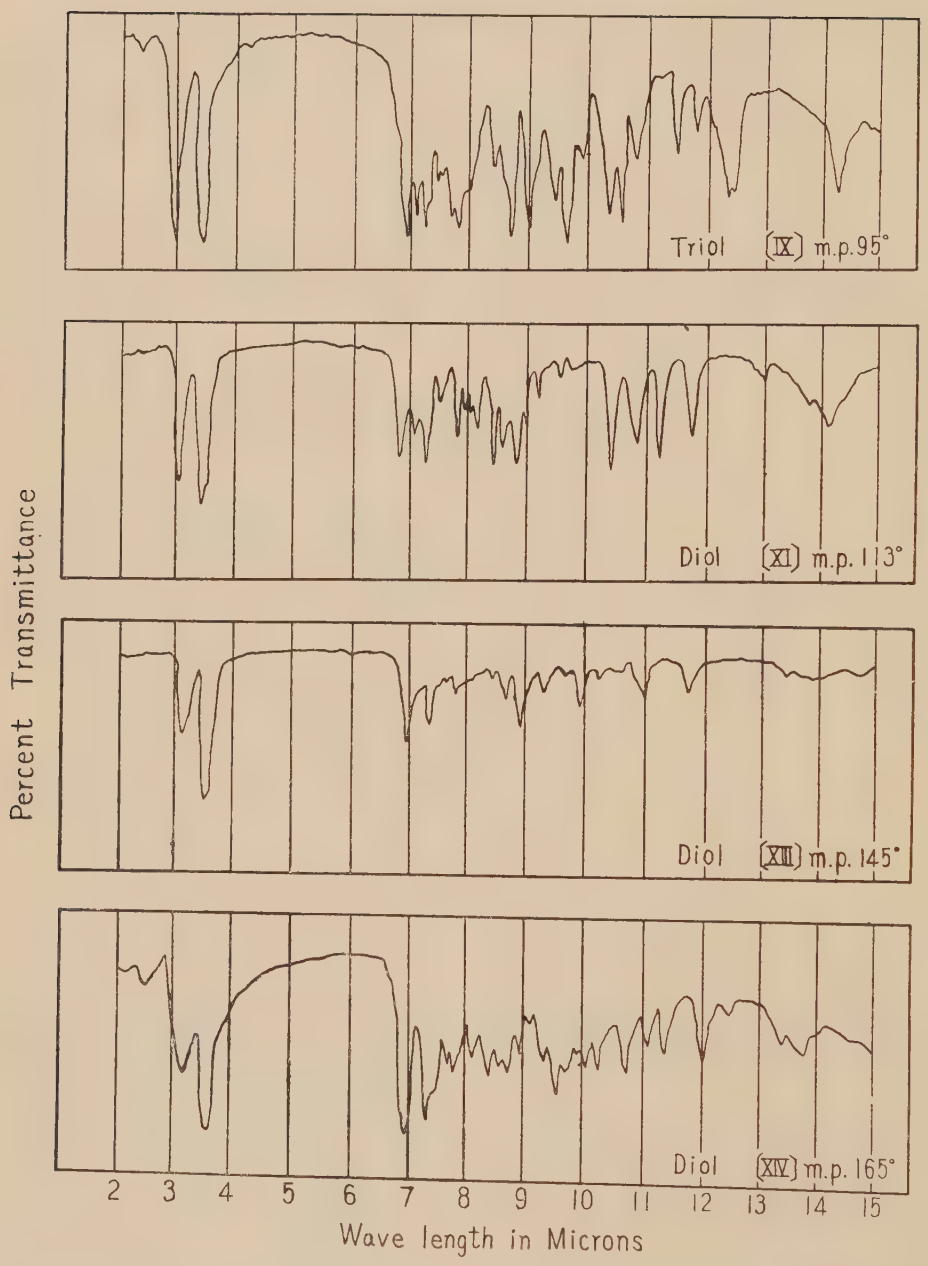
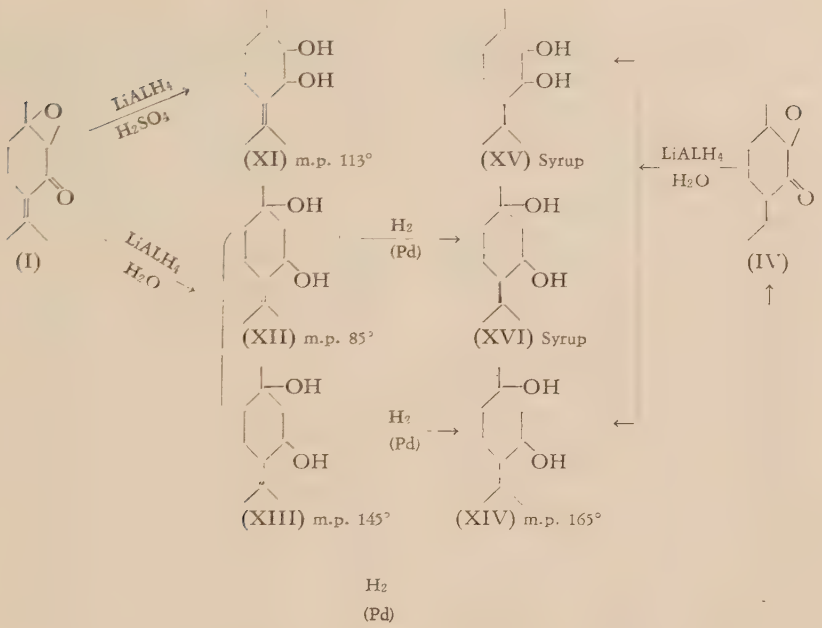


FIG. 2. Infrared Absorption Spectra of Diols and Triol derived from Rotundifolone (as Nujol mull).

original ketone, its epoxide and diol coincided completely with those of our rotundifolone series. Consequently, their ketone seems to be identical with our rotundifolone. However, the mintglyoxal formula



presented by them can not be accepted from our experimental results. In 1956, R. Reitsema et al.[7] reported on the isolation of a new terpenic ketone $C_{10}H_{16}O_2$, which has the structure of *l*-piperitone oxide. Many chemical and physical properties of dihydro-rotundifolone were found to coincide well with that of *l*-piperitone oxide. This fact gives support that the formula of rotundifolone is 1-methyl-4-isopropylidene-1,2-epoxy-cyclohexanone-3.

EXPERIMENTAL

All melting and boiling points were uncorrected. Ultraviolet absorption spectra were determined in methanol on a Beckman Model DU quarz spectrophotometer. Infrared spectra were recorded in Nujol on a Perkin Elmer double-beam spectrophotometer. Microanalyses were carried out by the Microanalytical Division, Prof. Mitsui's Laboratory, University of Kyoto.

Hydrogenation of the Oil Regenerated from (I)-Semicarbazone: The enolic oil (500 mg) which was regenerated from (I)-semicarbazone by treatment with 5% oxalic acid, had an absorption maximum at 255 mμ in the ultraviolet region, as shown in the previous report.[1] The regenerated oil [II'] was laevorotatory $[\alpha]_D^{10}$ −18.3° (c 1; MeOH) and absorbed hydrogen in the presence of palladium-barium sulfate (0.7 mol. equivalent of double bond) in 30 min.. Removal of the solvent and the catalyst left the oil, which soon solidified. It had an intense absorption maximum (λmax 273 mμ) and gave a dark green color by ferric chloride test.

When the alkali soluble-part of this oil was acidified by adding 2 N hydrochloric acid dropwise, a needle crystal (50 mg) was obtained. After vacuum sublimation and recrystallization from dilute methanol, it melted at 82° and did not show any depression by mixed m.p. with the authentic diosphenol[2]. This enolic substance had λmax 273 mμ (log ε=3.9 in MeOH) and coincided with the absorption spectrum[8] of authentic diosphenol. *Anal.* Found: C, 71.89; H, 9.88. Calcd. for $C_{10}H_{16}O_2$: C, 71.39; H, 9.59%.

Isolation of $C_{10}H_{18}O_3$ (VIII): The alkali insoluble-part of the hydrogenated oil mentioned above, was distilled under diminished pressure. The fraction 100–120° (6–7 mm. Hg) was recrystallized repeatedly from petroleum ether to give a fine needle of ultimate m.p. 85°, which showed a negative ferric chloride

7) R.H. Reitsema and V. J. Varnis, *J. Am. Chem. Soc.* **78**, 3792 (1956). The dihydro-rotundifolone semicarbazone was sent to Dr. R. Reitsema. It was found that there was no depression in melting point of our sample when mixed with *l*-piperitone oxide semicarbazone, isolated from the oil of *M. sylvestris*. The ultraviolet absorption also agreed. (Oct. 16, 1956 private communication).

8) Lowry and Lishmund, *J. Chem. Soc.* 1313 (1935); Gillam, Lynas-Gry, Penfold, and Simonsen, *J. Chem. Soc.* 62 (1941).

reaction and was related to (VIII). Yield 60 mg *Anal.* Found: C, 64.26; H, 9.79. Calcd. for $C_{10}H_{18}O_3$: C, 64.49; H, 9.74%. The weak absorption in the ultraviolet spectrum at wave length 277 mμ, (ε= 57.6 in MeOH) supported the existence of a saturated ketone group but further investigations could not be continued on account of its small amount.

Catalytic Hydrogenation of Rotundifolone (I): Rotundifolone (532 mg) was dissolved in methanol (30 ml) and hydrogenated by using palladium-barium sulfate (200 mg) as a catalyst. It absorbed 92 ml (12°, 701 mm. Hg) of hydrogen (equivalent to 1.0 double bond), and gave dihydro-rotundifolone which had a menthone-like odor. b.p. 90–95° (2-3 mm. Hg) $[\alpha]_D^{15}$ −142.5° (c 1.0; MeOH), n_D^{27} 1.4607, d_4^{27} 0.9842. It did not show any ferric chloride reaction, nor had it intense absorption maximum in the ultraviolet region. It yielded a monosemicarbazone in pyridine methanol solution. After recrystallization from ethylacetate, it decomposed at 210°; λmax 224 mμ ($\log\varepsilon$= 4.07) and λmax 243 mμ ($\log\varepsilon$=4.05, in MeOH). *Anal.* Found: C, 58.95: H, 8.36. Calcd. for $C_{11}H_{19}O_2N_3$: C, 58.64; H, 8.56%.

Rearrangement of Dihydro-rotundifolone (IV) to Diosphenol (III): Dihydro-rotundifolone semicarbazone (1.7 g) was refluxed with an aqueous solution (50 ml) of oxalic acid (1.5 g) for 30 min.. The resulted solution was cooled and extracted with ether continuously, for 10 hrs.. Evaporation of ether left the oil, which had two absorption (λmax 235 mμ, λmax 273 mμ), and gave a positive ferric chloride reaction. By acidifying the alkali soluble-part of this enolic oil, diosphenol (III) was obatined by the same method as described above. While dihydro-rotundifolone (IV) did not isomerise to an enolic substance by refluxing in neutral media, (IV) gave an enolic oil in acid media; it had the same ultraviolet spectrum as that of the oil regenerated from (IV)-semicarbazone. The absorption maximum at 273 mμ indicated the formation of diosphenol, separated in the same way as described before. Yield about 20%.

The Triol (IX) (m.p. 95°): Catalytic hydrogenation of the alkali insoluble-part (V) (500 mg) of the oil regenerated from (IV)-semicarbazone in the presence of PtO_2 (hydrogen absorption 64 ml), gave long needle crystals, which melted at 95° after repeated recrystallization from petroleum ether. Yield 220 mg. *Anal.* Found: C, 64.42 H, 10.91 Calcd. for $C_{10}H_{20}O_3$: C, 64.58; H, 10.84%. M.W. by the Rast's method 195; Calcd. for $C_{10}H_{20}O_3$ 188.

The compound (IX) was soluble in water, methanol and ethanol, but hardly dissolved in petroleum ether. It consumed 1.2 mol. of periodic acid in an aqueous solution in the course of 10 hrs., yielding a carbonylic substance, of which 2,4-dinitro-phenylhydrazone was a yellow substance (m.p. 144–149°). The oil (X), obtained from (IX) by treatment with dilute sulfuric acid was shown to be positive by the ferric chloride test, which supported the existence of a tertiary alcohol group in (IX).

Isorotundifolone (XVII) from (I)-Semicarbazone: Rotundifolonesemicarbazone (1.7 g) was refluxed with 5% sulfuric acid (100 ml) for one hour. The resulted oil had two intense absorptions i.e., at λmax 306 mμ as well as at λmax 255 mμ, while the regenerated oil by decomposition with oxalic acid showed one maximum. The distillate 90–100° (3 mm. Hg) crystallized and melted at 79° after recrystallization from dilute methanol; Colorless needle. λmax 306mμ ($\log\varepsilon$=3.95). Yield, 400 mg. It gave a positive ferric chloride reaction and was demonstrated to possess the molecular formula of $C_{10}H_{14}O_2$, from the analytical result. *Anal.* Found: C, 72.11; H, 8.71. Calcd. for $C_{10}H_{14}O_2$: C, 72.26; H, 8.49%.

As this enolic substance is an isomer of rotundifolone, it was named "iso-rotundifolone". It absorbed 3 molar equivalent hydrogen in the presence of PtO_2 to give *p*-menthandiol-2,3 which was identical with one of the reduction products of diosphenol. Further investigation will be reported in the near future.

Epoxide (VI) and Diol (VII) Derived from (I): To the ice cooled-solution of (I) (800 mg) in chloroform (30 ml), a cooled chloroform solution (30 ml) containing 2 g of perbenzoic acid was added. After the reaction mixture was kept at 0° for 24 hrs, the chloroform solution was washed with 5% sodium carbonate and dried over sodium sulfate. Removal of the solvent under diminished pressure gave a solid, from which a colorless crystal (VI) (m.p. 78°) was obtained after recrystallization from a mixture of ether and *n*-hexane. Yield, 500 mg. $[\alpha]_D^{10}$ −28.2° (c 8.0; MeOH). *Anal.* Found: C, 66.15: H, 7.55. Calcd. for $C_{10}H_{14}O_3$: C, 65.91; H, 7.74%.

The ultraviolet spectrum of epoxide, did not show any intense absorption. To 100 ml of 1% sulfuric acid, 500 mg of (VI) was added under stirring at room temperature (25°). After 30 min. the reaction solution became clear. The solution was stirred for 3 hrs., and then extracted with ether continuously, for 10 hrs..

The ether solution was dried over sodium sulfate completely and evaporated to give a solid. After the oil substance was separated, the residual solid recrystallized from ether, a needle crystal (VII), was obtained (m.p. 136°); yield, 200 mg. $[\alpha]_D^{15}$ −210° (c 0.78; H_2O). *Anal.* Found: C, 60.15; H, 8.15. Calcd. for $C_{10}H_{16}O_4$: C, 59.98; H, 8.06%.

The infrared spectrum of (VII) showed the presence of the hydroxyl group (3310 cm^{-1}) and carbonyl (1773 cm^{-1}), of which the band position of carbonyl was remarkably shifted to a short wave length, as compared with those of ordinary ketones. The diol (VII) 50 mg consumed only 0.5 mol. of periodic acid during the course of 24 hrs.. In this oxidation reaction, acetone was confirmed as 2,4-dinitro-phenylhydrazone (m.p. 124°).

Lithium Aluminium Hydride Reduction of Rotundifolone (I) (Decomposition in acid media): The diol (XI) (m.p. 113°): Rotundifolone (750 mg) dissolved in 30 ml of absolute ether was added dropwise to a cold (−10°) solution of lithium aluminium hydride (0.50 g), in 50 ml of absolute ether under effective stirring free from moisture. Upon the completion of addition, the solution was stirred at 0°, subsequently for one hour.

Then, stirring was continued at room temperature (10–15°) for 10 hrs.. Finally, the reaction solution was refluxed on the water bath and cooled. Excess hydride was decomposed by the addition of 50 ml of ether saturated with water under cooling and then treated with 20 ml of 10% sulfuric acid to remove aluminium hydroxide. The ether layer was separated, and the aqueous solution was extracted with ether continuously; then both ether solutions were combined. The evaporation of the solvent left a solid mass, which turned to long needle crystals of ultimate m.p. 113° after repeated recrystallizations from petroleum ether. Yield, 300 mg. $[\alpha]_D^{10}$ −7.2° (c 1.57; MeOH). *Anal.* Found: C, 70.33; H, 10.92. Calcd. for $C_{10}H_{18}O_2$: C, 70.54; H, 10.66%.

It consumed two mol. of periodic acid in the aqueous solution at 40°, thereby, the formation of acetone was confirmed as 2,4-dinitro-phenylhydrazone. These results indicated that the reduced product was a diol possessing one double bond, which is related to (XI). The infrared spectrum of this substance supported the diol constitution. (Fig. 2).

Lithium Aluminium Hydride Reduction of (I) (Decomposition in neutral media): The lithium aluminium hydride reduction was carried out according to the same procedure as that described above, except that 50 ml of water was used for hydrolysis in place of dilute sulfuric acid. After the ether readily soluble-part being filtered off and recrystallized from petroleum ether, it produced a fine needle (m.p. 145°) (XIII). Yield, 50 mg. $[\alpha]_D^{10}$ - 58.0° (c 0.5: EtOH). *Anal* Found: C, 70.23: H, 10.42. Calcd. for $C_{10}H_{18}O_2$: C, 70.54: H, 10.66%. Being catalytically hydrogenated, (XIII) absorbed one molar hydrogen and yielded a needle crystal (XIV) (m.p. 165°), which was hardly soluble in ether and was the same substance as that obtained from (IV), by lithium aluminium hydride reduction.

From the ether soluble-part of the reduction products, fine needles were separated and recrystallized from *n*-hexane to yield a diol (XII) (m.p. 85°). Yield, 200 mg. *Anal.* Found: C, 70.60; H, 10.57. Calcd. for $C_{10}H_{18}O_2$: C, 70.54; H, 10.66%.

It absorbed one molar equivalent of hydrogen over a palladium catalyst to give a syrupy dihydrodiol (XVI) which consumed no periodic acid. Therefore, this diol was related to (XII), which was a steroisomer of (XIII).

Lithium Aluminium Hydride Reduction of Dihydrorotundifolone (IV): Dihydro-rotundifolone (IV) (800 mg) was reduced by the action of lithium aluminium hydride (700 mg) according to the procedure described in the preparation of (XIII). From the hardly soluble-part in petroleum ether, laevorotatory needle (XIV) (m.p. 165°) was obtained after recrystallization from a mixed solvent composed of ether and petroleum ether. $[\alpha]_D^{25}$ −33.0° (c 0.96: MeOH). Yield, 180 mg. *Anal.* Found: C, 69.57; H, 11.87. Calcd. for $C_{10}H_{20}O_2$: C, 69.72: H, 11.70%. This laevorotatory substance consumed no periodic acid and was clearly confirmed to be 1,3-diol (XIV). The infrared spectrum of (XIV), showed the presence of the hydroxyl group but not of the carbonyl group.

The oil which was separated from (XIV), was distilled under vacuum. The main fraction (XV), boiled at 115–125° (1–2 mm. Hg). Yield 250 mg. $[\alpha]_D^{25}$ +26.2° (c 3.82; MeOH), n_D^{27} 1.4662, d_4^{27} 0.9715, MR_D 49.05 (Calcd. as $C_{10}H_{20}O_2$ diol 49.07). *Anal.* Found: C, 69.14; H, 11.95. Calcd. for $C_{10}H_{20}O_2$: C, 69.72; H, 11.70%. The diol (XV), consumed one molar equivalent of periodic acid in the course of 2 hrs. and was found to be an α-glycol.

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Electrolytic Reduction of Physiologically Important Substances at Controlled Potential

Part I. A Partially Active Product of Diphosphopyridine Nucleotide.

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By means of the electrolytic reduction of diphosphopyridine nucleotide at controlled potential using mercury as the cathode, the author has obtained a product which showed an absorption spectra similar to that of dihydrodiphosphopyridine nucleotide. The optical density of the product at 340 mμ diminished partly with the addition of alcohol-dehydrogenase and acetaldehyde or with that of malic acid-dehydrogenase and oxaloacetate as the oxidizing system. The same absorption band was completely disappeared by the addition of a fraction obtained from mung bean seedlings.

INTRODUCTION

Diphosphopyridine nucleotide (DPN) is a biologically important factor in the respiration system. The oxidized form of DPN (DPN^+) is reduced to dihydrodiphosphopyridine nucleotide (DPNH) either with a suitable dehydrogenase system or with sodium dithionite[1]. DPN has a sharp absorption band in the ultra-violet region at 260 mμ, while in DPNH, the height of this band is somewht reduced, and a second band, a rather broad one, makes its appearance at about 340 mμ. Substances with the absorption spectra similar to DPNH are known to be obtained from DPN^+. They are 1) an addition compound of DPN^+ with hydrogen cyanide[2,3], 2) an addition compound of DPN^+ with sodium dithionite[4,5], 3) a compound which is obtained by the X- or γ- ray irradiation of a diluted alcoholic solution of DPN^+[6,7], and 4) a reduced product of DPN^+ with borohydride[8].

Of these substances, 1) and 2) obviously have no relation to DPNH, but 3) and 4) are said to be the reduced products of DPN^+ having the same chemical formulae as DPNH. But the preparation of 3) is completely inactive towards alcohol- or lactic-dehydrogenase, and that of 4) is half as active as the enzymatic product.

These facts were explained by Stein and Stiassny as follows[7]: By the ionic reduction of DPN^+ with an enzyme or with sodium dithionite, addition of a hydrogen atom occurs at position 4 of the pyridine ring as it was proved by Pullman[9], But by the electronic reduction as occurs in the case of the irradiation, hydrogen may attach itself to position 2 or 6 of the pyridine ring giving a product which has no enzymatic activity. Finally by the reduction with borohydride, both reactions would proceed concomitantly, giving a mixture of the two reduction products.

On the other hand, polarographic studies of DPN revealed that it could be reduced electrolytically. But the half-wave potential of the DPN system obtained with a mercury cathode was reported to be -0.927 volt vs.

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S.C.E. (saturated calomel electrode) at pH 7.38 by Kaye and Stonehill[10] and −0.98 volt vs. S.C.E. at pH 10.3–10.6 by Carruthers and Tech[11]; while the standard oxidation-reduction potential vs. N.H.E. (normal hydrogen electrode) of the DPN system in enzymatic reaction was calculated as −0.325 volt at pH 7.4 by Clark[12], and −0.318±0.003 volt at pH 7.0 (30°C) by Rodkey[13], which roughly corresponds to −0.57 volt against S.C.E.. The discrepancy of the data was not explained until Stein and Stiassny suggested that the electrolytic reduction of DPN^+ would proceed electronically, producing an enzymatically inactive compound.

The author actually reducing DPN^+ by the method called "electrolytic reduction at controlled cathode potential"[14-17], found out that the ultraviolet spectrum of the product was similar to that of DPNH[18], and the height of the absorption band at 340 mμ diminished partially by enzymatic oxidations, although attempts to increase the percentage of the enzymatically active component were not fruitful.

While these studies were in progress, a similar experiment was performed by Ke[19,20], who reported that the electrolytically reduced DPN was totally inactive towards alcohol- or lactic-dehydrogenase. His result was consistent with the theory of Stein and Stiassny, but inconsistent with the author's result. The author repeated the experiment in various conditions to confirm his conclusion, that even the electrolytically reduced DPN is enzymatically active to some extent at least. In the course of the experiment, it was also observed that the absorption band at 340 mμ of the electrolytically reduced DPN solution completly disappeared on addition of a fraction obtained from mung bean seedlings.

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EXPERIMENTAL

Apparatus. The electrolytic reduction of DPN^+ at controlled cathode potential was performed in the cell with a sintered glass boundary between the cathode and anode solutions (Fig. 1). Platinum plate and mercury were used as the anode and cathode, respectively. The cathodic solution was stirred efficiently with a magnetic stirrer at room temperature, while purified nitrogen was passed through the solution. The electric circuit of the apparatus is shown in Fig. 2.

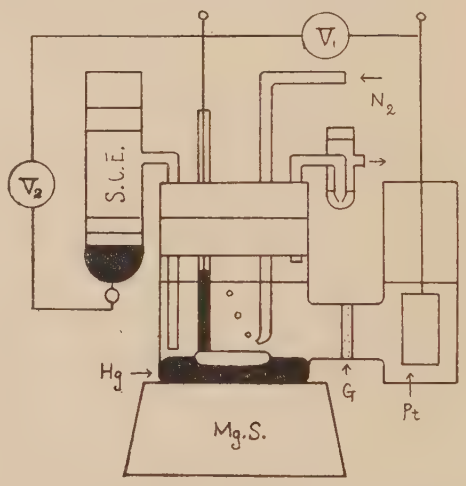

FIG. 1. Apparatus Designed for the Electrolytic Reduction at a Controlled Cathode Potential.
Mg. S.: magnetic stirrer, G: glass filter.

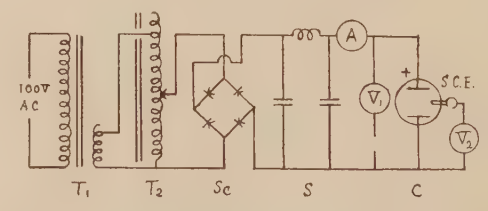

FIG. 2. Circuit for Electrolyic Reduction at a Controlled Cathode Potential.
T_1: transformer, T_2: slide-transformer, Se: rectifier, S: stabilizer, C: elcetrolytic cell (Fig. 1).

Reagent. DPN of about 90% purity, obtained from National Biochemicals Co., U.S.A., was used in the typical experiments. DPN of about 30% purity, prepared after the method of LePage[21], was also used in some part of the experiments.

Alcohol dehydrogenase was prepared according to the method of Racker[22].

Malic acid dehydrogenase was prepared after the method of Straub[23], and a crude product obtained from pig heart by acetone fractionation was used.

The fractionation of etiolated mung bean seedlings was made in the following way. Etiolated mung bean seedlings, cultured for four or five days, were homogenized with a half of its weight of 0.1 M Na_2HPO_4 solution. The extract was half saturated with ammonium sulfate, the resulting precipitate was collected and dissolved in one-fifth of the original volume of 0.1 M phosphate buffer pH 7.2.

Method. The reduction of DPN^+ with sodium dithionite was carried out as follows. About 2.5 mg of DPN^+ was dissolved in 5 ml of 0.1 M phosphate buffer, pH 7.2. To the solution, 0.5 ml of 3% sodium bicarbonate solution containing 3% sodium dithionite was added. After the mixture was left for 20 mins., 4.5 ml of the phosphate buffer was added, and the solution was aerated for 5 mins. Then the solution was filled up to 50 ml with phosphate buffer, pH 7.2.

The electrolytic reduction of DPN^+ was carried out as follows. About 2.5 mg of DPN^+ was dissolved in 25 ml of 0.1 M phosphate buffer, pH 7.2, and was reduced at −1.75 volt vs S.C.E. for 30 mins. in the apparatus shown in Fig. 1. Then the solution was filled up to 50 ml with phosphate buffer, pH 7.2.

Measurement of the optical density of the solution was made with a Beckman's Spectrophotometer Model DU. The enzymatic reactions were followed as the change in optical density (ΔE_{340}) in a spectrophotometric cell with a 1-cm light pass. Every enzymatic reaction was studied at pH 7.2 at room temperature.

RESULTS

Electrolytic Reduction of DPN. Ultra-violet absorption spectra of electrolytically reduced DPN solution (DPNH-el.), reduced at various cathode potentials are illustrated in Fig. 3. Hydrogen gas did not generate until at −1.75 volt (vs. S.C.E.), and the pH of the solution was kept constant within 0.1 during the course of the reduction. At −1.80 volt, however, hydrogen gas appeared on the cathode, and the pH of the solution increased from 7.2 to 7.4 in 30 mins. during the reduction. The ultra-violet absorption spectra of DPN^+, DPNH-el. (reduced at −1.75 volt for 30 mins.), and DPNH obtained by the reduction with sodium dithionite (DPNH-di.) are shown in Fig. 4. The changes of the electric current and the cathode potential during the reduction are shown in Fig. 5.

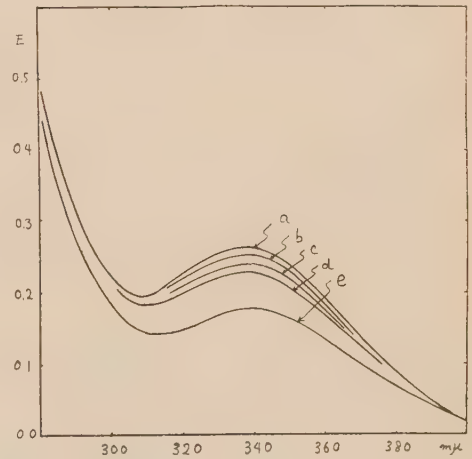

FIG. 3. Ultra-violet Absorption Spectra of Electrolytically Reduced DPN.

a: reduced at −1.8 volt, for 30 mins., b: −1.75 volt, 30 mins., c: −1.6 volt, 60 mins., d: −1.4 volt, 60 mins., e: −1,2 volt, 60 mins.

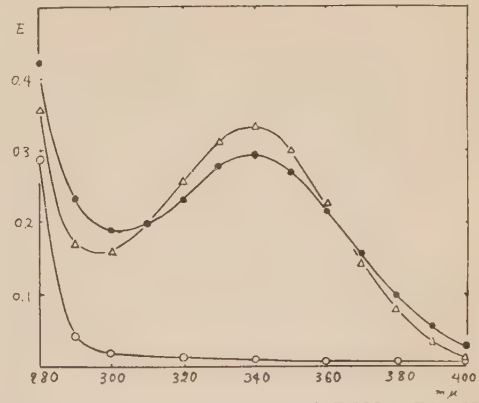

FIG. 4. Ultra-violet Spectra of DPN^+, DPNH-di. and DPNH-el.

○—○ DPN^+, △—△ DPNH-di., ●—● DPNH-el.

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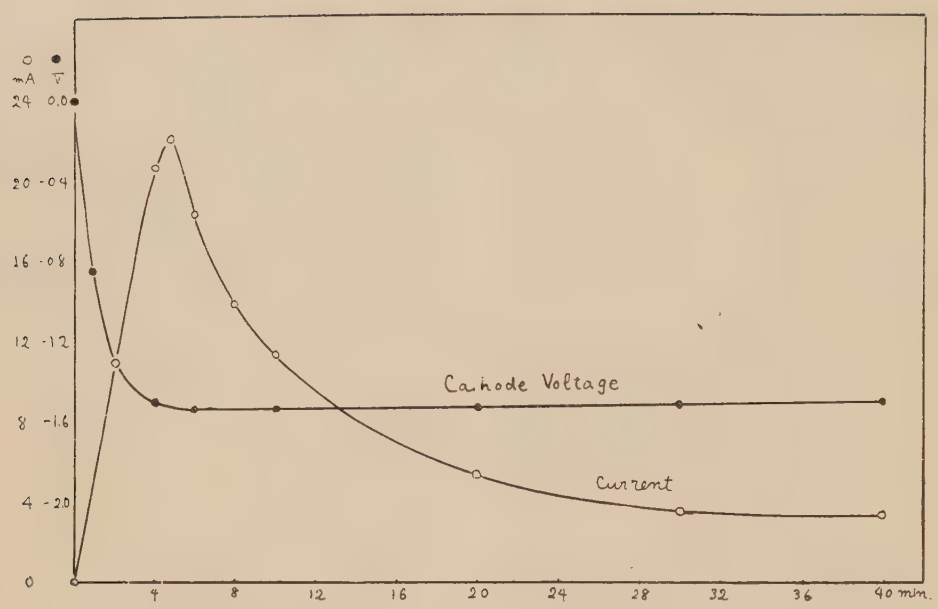

FIG. 5. Electric Current and Cathode Potential During Reduction.

The Reaction with Alcohol Dehydrogenase. The reactions of DPNH-el. and DPNH-di. with alcohol dehydrogenase were compared. An appropriate amount of enzyme was added to 10 ml of the DPNH solution and 3 ml of the solution was taken in a spectrophotometric cell. The same amount of the enzyme was dissolved in 10 ml of the buffer solution, and 3 ml of the solution was taken in another cell as the control. The decrease of the optical density at 340 mμ was measured after the addition of 0.1 ml portions of 1 M acetaldehyde solution both into the DPNH solution and the control. Typical examples of the reaction are shown in Fig. 6 and 7; DPN of 90% purity was used in the experiment. It was observed that about one-third of the electrolytically reduced DPNH (DPNH-el., reduced at -1.75 volt) was active towards alcohol dehydrogenase. Of course no reaction took place without the enzyme. The possibility of the contamination of the DPNH-el. solution by the inhibitory mercuric ion was excluded by the following experiment. Namely, no inhibitory action

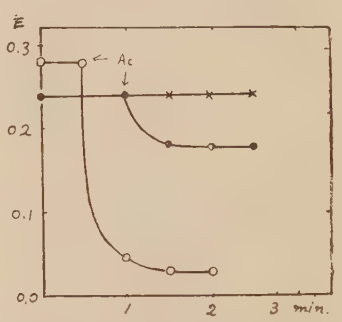

FIG. 6. Reaction of Alcohol Dehydrogenase.
○—○ with DPNH-di., ●—● with DPNH-el., and ×—× with DPNH-el. without acetaldehyde. Ac. → indicates the addition of acetaldehyde solution.

was observed in the enzymatic oxidation of DPNH-di., filled up to 50 ml with the addition of 25 ml of the buffer solution which had been subjected to the reduction procedure. The results are shownin Fig. 8.

The ratio of the enzymatically active component in DPNH-el. was affected neither by the purity of the DPN preparation, nor the change of the cathode potential in the range from -1.40 to -1.75 volt.

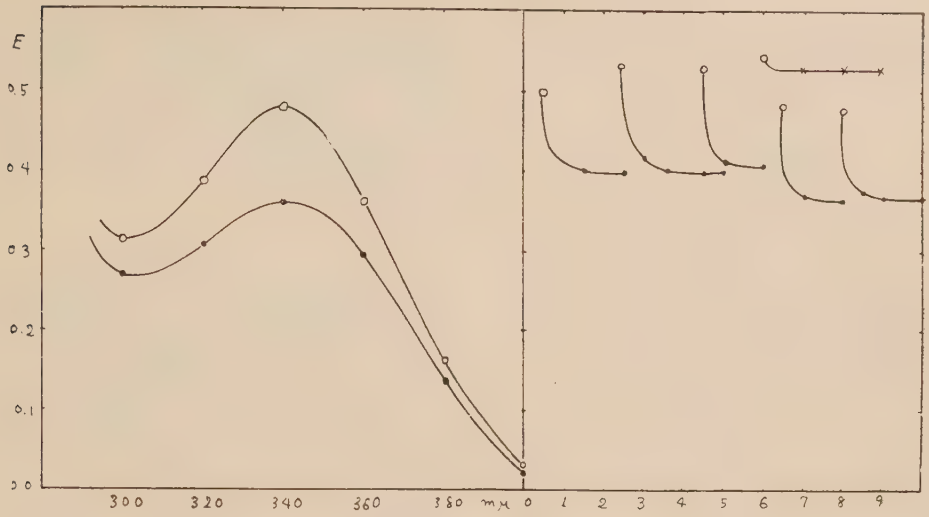

FIG. 7-a. Absorption Spectra before and after the Addition of Acetaldehyde to the Mixture of DPNH-el. and Alcohol Dehydrogenase.

FIG. 7-b. Changes of Optical Densities at 340 mμ after the Addition of Acetaldehyde Solution to either ○—● a mixture of DPNH-el. and Alcohol Dehydrogenase, or ○—× DPNH-el. Solution.

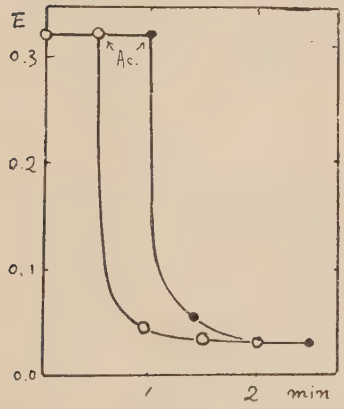

FIG. 8. Effect of the Buffer Subjected to the Reduction Process, on the Reaction of DPNH-di. with Alcohol Dehydrogenase and Acetaldehyde.

● —● DPNH-di. of 25 ml was diluted with 25 ml of the buffer subjected to the electrolytic reduction process. ○—○ Control.

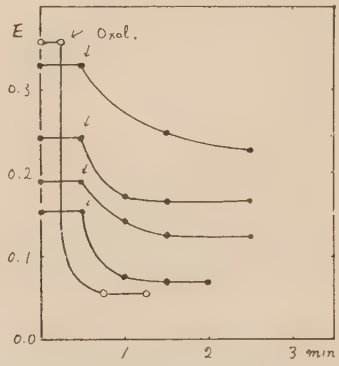

FIG. 9. Reaction of Malic Acid Dehydrogenase. ○—○ with DPNH-di. and ● —● with DPNH-el.

The Reaction with Malic Acid Dehydrogenase. Experiments of the same kind as described above were conducted using oxaloacetate and a crude preparation of malic acid dehydrogenase. The results are shown in Fig. 9. As in the case of alcohol dehydrogenase, from one fourth to one-third of DPNH-el. (reduced at −1.75 volt) was active towards the enzyme, while DPNH-di. was fully active as it was expected. The DPN preparation of about 30% purity was used in this experiment.

The Reaction with a Fraction of Etiolated Mung Bean Seedlings. A fraction obtained from etiolated mung bean seedlings by ammonium sulfate fractionation was added to the DPNH solution, and the change of the optical density at 340 mμ was measured. It was observed

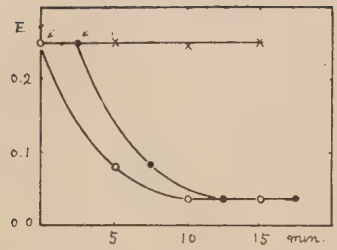

FIG. 10. Rcaction of the Fraction Obtained from Mung Bean Seedlings.

○—○ with DPHN-di., ●—● with DPNH-el., ×—× reaction of 10mins. boiled-fraction.

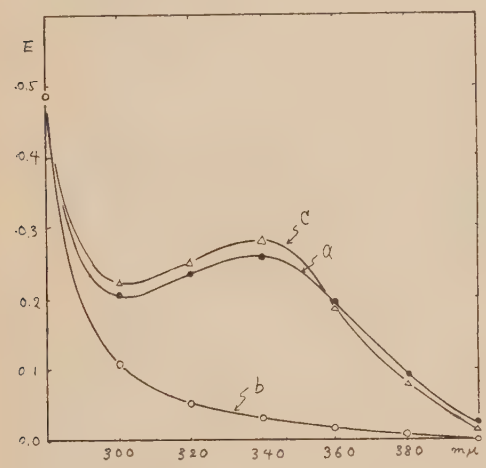

FIG. 11. Effect of the Fraction Obtained from Mung Bean Seedlings on DPNH-el.

Absorption band of DPNH-el. (a) diminished by the addition of the fraction (b), but appeared again (c) by the reduction of (b) with sodium dithionite.

that by the addition of 0.1 ml of the (enzyme) solution, the optical densities of both the DPNH-di. and DPNH-el. solutions are diminished in almost the same way as it is shown in Fig. 10. Since the fraction lost its activity by boiling, the effect was supposed to be enzymatic. At least, the pyridine ring in the DPN was not affected by the reaction, because by the reduction of the supposedly oxidized solution with sodium dithionite after deproteinization with basic lead acetate, the ultra-violet absorption band at 340 mμ made its appearance again. The results are shown in Fig. 11.

CONCLUSION

As explained in the introduction, electrolytically reduced DPN had been reported to be totally inactive towards alcohol- or lactic dehydrogenase. But the author has proved that the electrolytically reduced DPNH at a constant cathode potential, −1.75 volt vs. S.C.E., is active as much as from one-fourth to one-third at least towards alcohol- or mailc acid-dehydrogenase. A mercury cathode was used in the experiment. The same reduction product seemed to be completely oxidized with a fraction obtained from etiolated mung bean seedlings. Studies of this fraction in the seedlings is now in progress.

While preparing this paper, Ke's recent article[24] has come to hand, in which he reported that although the electrolytically reduced product of DPN formed on the mercury electrode possessed no enzyme activity, the reduction compound at a platinum or lead electrode showed partial activity. He reduced DPN^+ in 0.2 M tris-buffer pH 7.5, at −1.4 volt for 60 mins. The apparatus used for the reduction happened to be almost the same with that used by the author.

The author is grateful to Prof. S. Funahashi and Assist. Prof. M. Nakamura for their interest in this work.

24) B. Ke, *J. Am. Chem. Soc.*, **78**, 3647 (1956).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 2, p. 121~125, 1957]

A Micro Amperometric Titration of Thiol-Group in Plant Tissues by the Use of a Vibrating Platinum Electrode*

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A method for the micro amperometric determination of thiol-groups as small as from 0.1 to 1.0 μ mole, corresponding to 10–100 μ g of cysteine, was devised. The thiol-group was titrated in ammoniacal solution in the stream of nitrogen with silver nitrate solution, using an ultramicroburette. The course of the titration and the end point was detected amperometrically using platinum vibrating electrode. The method was suitable for the determination of a minute amount of thiol-groups, especially those found in plant tissues, which used to contain such substances as might otherwise disturb the determination. Contents of acid-soluble thiol compounds in etiolated mung bean seedlings at various stages of growth were determined by the method described here.

INTRODUCTION

A number of methods have been proposed for the determination of a small amount of thiol- (or sulfhydryl) groups contained in biological materials, especially for that of glutathione in animal tissues and of cysteine residues in protein[1,2,3].

Iodometry[4], amperometry[4] and colorimetry with sodium nitroprusside[6] were the methods most prevailed, while the enzymatic[7] or microbiological[8] method was used in special cases. Potentiometry[9], polarography[10] and special colorimetries[11,12] were also involved. Of these, however, the amperometric method seems to be the most suitable for the determination of thiol-groups in plant tissues, because plant extracts are colored in most cases, turbid in some occasions, and usually contain reducing substances, which may otherwise disturb the determination. The amperometric titration which is indifferent to the turbidity or color, may be specific to thiol-groups provided that an appropriate reagent is selected.

Detailed studies on the amperometric determination of thiol-groups in amounts larger than 1 μ mole were made by Kolthoff and Stricks[13,14] and Hata[15,16] using a rotating platium electrode. Rosenberg et al.[17] tried to determine a minute amount of thiol-groups as small as 0.1 μ mole by using the vibrating platinum electrede, but they neglected the autoxidation of the thiol-groups by the air.

This paper presents a method for the accurate determination of thiol-groups ranging from 0.1 to 1.0 μ mole, corresponding to 10–100 μ g of cysteine. The use of a small

* The present work was aided by a grant from the Scientific Research Fund of the Ministry of Education.

1) F.C. Chinard and L. Hellerman, Methods of Biochemical Analysis. Vol. 1, p. 1 (1954).
2) W.J. Paterson and A. Lazarow, *ibid.* Vol. 2, p. 259 (1955).
3) E.S.G. Barron, *Advances in Enzymology.* Vol. **11**, p. 201 (1951).
4) A. Fujita and I. Numata, *Biochem Z.* **299**. 249 (1938).
5) R.E. Benesch et al., *J. Biol. Chem.* **216**, 663 (1955).
6) R.R. Grunert and Phillips, *Arch. Biochem. and Biophys.* **30**, 217 (1951).
7) E. Racker, *J. Biol. Chem.*, **190**, 685 (1951).
8) G.D. Shockman, *Anal. Chem.*, **26**, 1657 (1954).
9) R. Cecil, *Biochem. J.*, **47**, 572 (1950).
10) D.M. Coulson et al, *Anal. Chem.*, **22**, 525 (1950).
11) S.R. Bebedict and G. Gottschal, *J. Biol. Chem.* **99**, 729 (1933).
12) M.X. Sullivan and W.C. Hess, *J. Biol. Chem.*, **145**, 621 (1942).
13) I.M. Kolthoff and W. Stricks, *J. Am. Chem. Soc.*, **72**, 1952 (1950).
14) I.M. Kolthoff and W. Stricks, *Anal. Chem.*, **23**, 763 (1951)
15) T. Hata, *Bull. Research Inst. Food Sci. Kyoto Univ.*, No. **2**, 13 (1949).
16) T. Hata, *ibid.* No. **4**, 45 (1951).
17) S. Rosenberg et al., *Anal. Chm.* **22**, 1186 (1950).

amount of the solution and titration with an ultramicroburette in an atmosphere of nitrogen are essential features of this procedure.

EXPERIMENTAL

Apparatus. The apparatus is shown in Fig. 1. A controlled potential vs. S.C.E. was applied to the vibrating platinum electrode, and the difference of the conductivity of the solution at that potential during titration was read by a galvanometer. An ultramicroburette, which permit to measure 0.1 μl, was used[18]. Nitrogen gas was passed through the reaction mixture during the titration. The nitrogen was purified free from oxygen by passing through an alkaline pyrogallol solution and two buffer solutions successively, the latter have the same composition as that placed in the titration vessel.

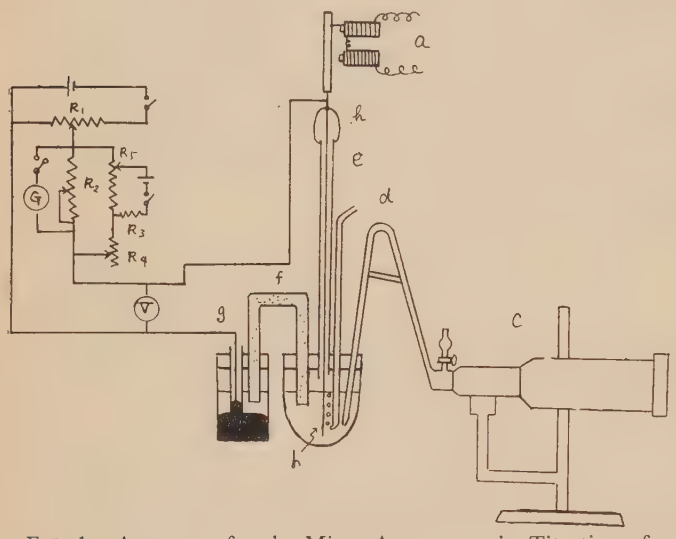

FIG. 1. Apparatus for the Micro Amperometric Titration of Thiol-Group.

a: elcctromagnet, b: platinum electrode, c: ultramicroburette, d: nitrogen inlet, e: nitrogen outlet, f: agar bridge, g: saturated calomel electrode, h: rubber nipple. R_1: 100 Ω, R_2: 1 kΩ, R_3: 3 kΩ, R_4: 50 kΩ, R_5: 50 Ω, G: galvanometer, V: voltmeter (1.5 volt).

Reagent. Compositions of the buffer solutions were: 0.1 M NH_4OH, 0.2 M NH_4NO_3 and 0.04 M Na_2SO_3 for the titration with 0.002 N silver nitrate solution, and 0.05 M NH_4OH, 0.1 M NH_4Cl and 0.1 M Na_2SO_3 for that with 0.002 N cupric sulfate solution. The cupric sulfate solution was prepared after the method of Kolthoff and Stricks[14], but the air was not driven off the solution.

N-acetylcysteamine[19], butylmercaptan[20] and *o*-chloromercuriphenol[21] were prepared in the laboratory. Cysteine hydrochloride, thioglycolic acid and *p*-chloromercuribenzoate were purchased from Wakô Pure Chemical Ind. Glutathione was granted from Dr. Kuroiwa of the Kirin Research Institute.

Procedure. The potential of the platinum electrode was adjusted to −0.4 volt vs. S.C.E. Two ml of the buffer solution was placed in the vessel, and the air dissolved in the solution was expelled by passing nitrogen through it at the rate of about 60 ml/min. A sample solution, which was free from oxygen, was quickly added. The bubbling of nitrogen was kept continued throughout the titration to prevent the thiolgroups from oxidation and to agitate the solution. The platinum electrode was vibrated at an amplitude of about 3 mm. The galvanometer circuit was closed and the zero point adjusted. Galvanometer readings were made after each addition of 0.5 μl of the reagent. Titrations were carried out at room temperature.

RESULT

Titration of Test Materials. Typical titration curves with and without any thiol-compound are shown in Fig. 2. The silver nitrate solution was used in this experiment,

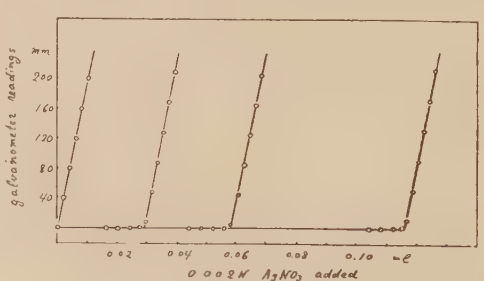

FIG. 2. Titration Curves.

0, 10, 20, and 40 μg of cysteine hydrochloride were titrated with 0.002 N silver nitrate solution.

18) N. Tanaka and M. Nishigai, *Report of Inst. of Sci. and Tech., Univ. of Tokyo* **6**, 131 (1952).

19) R. Kuhn and G. Quadbeck, *Ber.* **84**, 844 (1951).

20) Org. Synth. Collected Volume. II, p. 1045.

21) *ibid*. Collected Volume. I, p. 155.

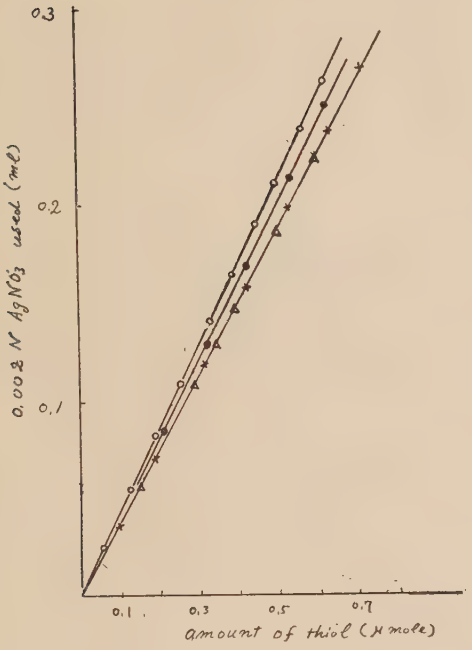

FIG. 3. Titrations of Thiols with Silver Nitrate Solution.
○——○ cysteine hydrochloride, ●——● thioglycolic acid, ×——× glutathione, △ —△ N-acetylcysteamine.

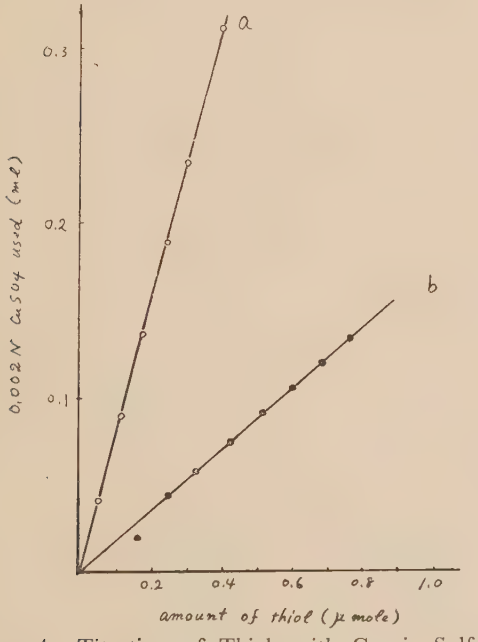

FIG. 4. Titrations of Thiols with Cupric Sulfate Solution.
a: cysteine hydrochloride, b: N-acetyl cysteamine.

but almost the same types of curves were obtained when the cupric sulfate solution was used as the titrating agent. Results of titrations of several test materials in various amounts with either the silver nitrate or the cupric sulfate solution are summarized in Figs. 3 and 4. Each spot in the figures indicates the mean value of three determinations. Standard deviations of the tirations are shown in Table I. Volatile compounds such as butylmercaptan could not be titrated by this procedure, because of the bubbling of nitrogen.

TABLE I
STANDARD DEVIATIONS OF THE TITRATIONS

	with 0.002 N $AgNO_3$		with 0.002 N $CuSO_4$	
	mean value $\bar{x}$, ml	standard deviation $\hat{\sigma}$, ml	mean value $\bar{x}$, ml	standard deviation $\hat{\sigma}$, ml
cysteine hydrochloride	0.146	0.0012	0.230	0.0083
N-acetyl-cysteamine	0.164	0.0014	0.086	0.0013

Figures indicate the results of eleven determinations.

Although both reagents gave satisfactory results in the experiment as shown in Table I, the cupric sulfate solution was apt to give irregular results in routine work, and its equivalent amounts for different thiol-groups differed rather considerably as shown in Fig. 4. Hence, the silver nitrate solution alone was used as the titrating agent in the following determinations.

Inhibitors and Masking Reagents. The effects of substances which might disturb the titration with the silver nitrate solution were examined. The results obtained were similar to those obtained by other investigators[13,15], the presence of sodium chloride less than 15 μ mole or that of 30 μ mole of potassium thiocyanate did not affect the titration. It was found that the addition of 3 to 5 equivalents of *o*-chloromercuriphenol masked thiol-groups completely, as in the case of addition of the *p*-chloromercuribenzoate[15].

Determination of Thiol-compounds in Some Plant Tissues. The acid-soluble thiol-compounds in

some plant tissues were titrated with the silver nitrate solution. The thiol-compounds were extracted with 5% sulfosalicylic acid following the method of Woodward and Fry[22]. Five grams of etiolated mung bean seedlings was homogenized with the addition of 40 ml of 50% sulfosalicylic acid, and the homogenate was centrifuged at about 100×g for 10 mins. The supernatant solution was then subjected to determination. In order to neutralize the sulfosalycilic acid present, an equivalent amount of 1 N sodium hydroxide solution was added to the titration mixture.

The amount of thiol-compounds in mung bean seedlings varied with the stages of growth, and the seedlings purchased on market did not contain any acid-soluble thiol-compound, in some occasions.

Recovery of the added glutathione in the acid-extract of the seedlings gave a value of 93% on the average, as shown in Table II.

The amounts of acid soluble thiol-compounds in mung bean seedlings at various stages of growth are summarized in Fig. 5. The mung bean was germinated in the dark at 25°, and the results were calculated as the amount of glutathione in 100 g of dry matter. Two maxima of the amount of the total acid-soluble thiol-compounds were observed on the second and the seventh day.

Distribution of the thiol-compounds in the seedlings are illustrated in Fig. 6.

TABLE II
RECOVERY TEST OF GLUTATHIONE*

glutathione found in the extract	glutathione added	glutathione recovered	recovery
0 μg	60 μg	55 μg	93%
0	70	66	95
0	85	80	94
16	80	74	93
32	80	72	90
		mean	93

* The amount of the extract used was 1 ml.

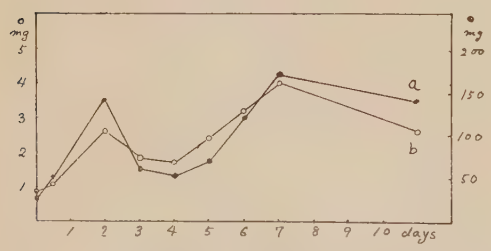

FIG. 5. Amounts of Thiol-compounds in Mung Bean Seedlings.
a: calculated as the amount of glutathione in 100 g dry matter, b: calculated as that in 100 seedlings.

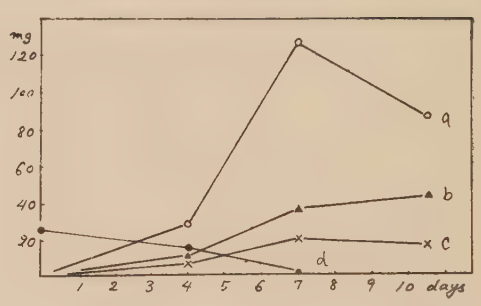

FIG. 6. Distribution of Thiol-compounds in Mung Bean Seedlings, the Results were Calculated as the Amounts of Glutathione in 100 g of Dry Seedlings.
a: hypocotyl, b: leaf, c: root, d: cotyledone.

DISCUSSION

Thiol-grups, ranging from 0.1 to 1.0 μ mole could be titrated amperometrically using a vibrating electrode with satisfactory results. The use of the vibrating electrode was effective in minimizing the amount of the solution to be titrated. The bubbling of nitrogen during the titration prevented the thiol-groups from oxidation. Titration with the silver nitrate solution was preferable, because slightly lower results were sometimes obtained in the titration with the cupric sulfate solution. This fact might be attributed to the oxidation, catalized by the cupric ion[4], of the thiol-groups by the molecular oxygen present in the cupric sulfate solution.

The appearance of thiol-groups in the seedlings had been observed by Vivario and Lecloux[23], and it was identified as glutathione

22) G.E. Woodward and E. Fry, *J. Biol. Chem.*, **97**, 465 (1932).

23) R. Vivalio and J. Lecloux, *Arch. Intern. Physiol.*, **32**, 1 (1930).

by Hopkins et al[24]. However, the distribution of Coenzyme A, a thiol-compound, in plant tissues was also reported recently[25]. Studies of the nature and role of the thiol-compounds in the seedlings are now in progress.

The authors are grateful to Dr. Yoshiro Kuroiwa of the Kirin Research Institute, for his generous gift of the sumple of glutathione.

24) F.G. Hopkins and E.J. Morgan, *Nature*, **152**, 288 (1943).
25) E. Seifter, *Plant. Physiol.* **29**, 403 (1954).

[Bull. Agr. Chem. Soc. Japan, Vol. 21, No. 2, p. 126~131, 1957]

The Inhibition of Nitrate Reduction with Silkworm Tissue

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Received October 18, 1956

In the tissue of silkworm the existence of an inhibitor of biological nitrate reduction has been demonstrated. This substance is heat-resistant and cannot be decomposed by autolysis. The inhibitor is contained, in the tissue of larvae, even in the acetone preparation, pupae, digestive juice and worm feces, but not in the blood and alimentary canal. When the worm is attacked with polyhedral virus disease or fed with nitrites, inhibiting ability of tissue is retained.

This inhibitor was isolated by extraction with diluted alkaline solution following precipitation at about pH 4.2. On the basis of ultraviolet absorption spectrum and Murexide reaction, the inhibitor was deduced to be uric acid. Thence, the inhibiting action of uric acid was comparatively studied. Both the inhibitor isolated and pure uric acid diminished their inhibiting capacity by the pretreatment of mouse liver containing uricase, but not so in the case of fowl which has not such enzyme.

The reducing ability of larval tissue was attested by the removal of this inhibitor.

It is known that nitrites are formed from nitrates by the catalytic action of the homogenate of some animal tissues[1]. But the reducing ability of tissue of silkworm, *Bombyx mori*, has not yet been observed with the pure lines—*P-21 and P-44*, and their mutual hybrides. Judging from our experiences, however, I somewhat hesitate to conclude that these strains of worm have not such ability. It might be supposed that the activities of tissue do not function under the condition employed on account of some reasons. I, therefore, intended to investigate the cause of this abnormality.

EXPERIMENTS AND RESULTS

Inhibiting Action of Worm Tissue on Nitrate Reduction. Nitrate reduction and inhibiting tests were carried out essentially, according to the previous paper[2]. It was, however, supposed that the colorimetric estimation of nitrites employing Griess's reagent might be hindered by worm tissue such as cysteine and ascorbic acid, or that nitrites might be further exhausted according to some reasons, e.g., nitrite reduction, as soon as it was formed. First, therefore, the recovery of nitrites which had been added to the reaction mixture was estimated. In place of nitrates, 10^{-4} M $NaNO_2$ solution was mixed with the homogenate of larval tissue which was freed from the alimentary canal, and nitrites were measured at both the beginning and end of the reaction. If the nitrites do not develop a red color at zero-time, the tissue will arrest estimation. When the decrease of nitrites added is observable, the nitrites will be further decomposed. However, it was found that such fear was unnecessary.

Then, the effect of worm tissue on nitrate reduction was examined using mouse liver extract which is the reducing material mainly employed, unless otherewise indicated. The liver was taken immediately after decaptitation and extracted with M/15 phosphate buffer of pH 6.0, in the usual manner. Table I. indicates the inhibiting effect of the boiled homogenate of worm tissue. It can be observed that the remarkable hindering action may be attributed to some substance contained in the larval tissue.

As it has been stated elsewhere, in the concern of bacterial nitrate reductase[3], it can be deduced that nitrate reduction proceeds through the transmission of

1) F. Bernheim and M. Dixon, *Biochem. J.*, **22**, 125 (1928).
2) H. Omura, *J. Fac. Agr. Kyushu Univ.*, **10**, 365 (1956).
3) F. Egami and R. Sato, *J. Chem. Soc. Japan*, **69**, 160 (1948)

Table I
Inhibition with Boiled Homogenate of Worm Tissue

Worm tissue added, g	none	0.2	0.1	0.05
NO_2 formed, μM/l	22.4	4.3	5.3	10.7
Inhibition, %		80.8	76.3	52.2
Time of M.B. decoloration, min.	18		37	30

Worm: *P-21*, 3rd day of the 4th instar. Mouse liver: 0.4 g.

hydrogen to nitrites in the liver also. The hydrogen may be provided from hydrogen donators by some relevant dehydrogenases. In fact, it was confirmed[4] that nitrate reduction with liver extract has close connection with several substances which can donate hydrogen in biological materials. If the supply of hydrogen is suppressed owing to the inactivation of dehydrogenases, the reduction of nitrates might also be retarded. As for an index of hydrogen donating ability, therefore, the methylene blue method was applied. A methylene blue solution of 10^{-4} M final concentration was substituted for the nitrates, and the time of decoloration was estimated under the same condition. In this case, also, a prolonged time was requirad by the addition of worm tissue, as presented in Table I.

Then, the tissue suspension of caterpillars was boiled and centrifuged. It is clear from Table II. that the inhibitor can be almost extracted. Therefore, all the following tests in this study were conducted with the boiled-extract of worm tissue. But the amounts of "worm tissue added" as indicated in the Tables mean that one which corresponds to the original fresh tissue. While, on the other hand, the above suspension was autolysed at 37°C for 20 hours with the addition of toluene, boiled and centrifuged. The clear supernatant, autolysate, thus obtained also exhibited inhibiting action. From the results shown in Table II., it seems likely that the inhibitor is not decomposed, but increased by autolysis.

In investigating the influences of chemicals upon nitrate reduction in vitro, attention must be payed, as already pointed out[2], on the fact that the effect of chemicals varied with the enzyme materials. So the retardations of nitrate reduction by worm tissue were examined with cattle liver and green algae. The cattle liver extract was also prepared in the same manner as that of mouse. Since acetaldehyde accelerates the reduction exceedingly, the effects of larval tissue on the reducing ability of cattle liver with and without 2×10^{-2} M aldehyde were tested. The data presented in Table III. indicate that, though worm tissue interferred with the enzymatic formation of nitrites from nitrates in both cases, hindrance was to some extent recovered, when reduction was greatly promoted by the addition of aldehyde.

The fact that the increase of reducing ability of animal tissue tends to decrease the inhibition with worm tissue might also be suggested from the experiments in which reducing capacity was changed by altering the amounts of liver used. Table IV. gives an example.

With regard to green algae, *Scenedesmus sp.*, isolated in our laboratory[5], suspension and extract of the resting cells were employed, according to the procedure

Table II
Effect of Extration and Autolysis of Worm Tissue

Part tested		extract		residue		autolysate	
Worm tissue added, g	none	0.1	0.05	0.1	0.05	0.1	0.05
NO_2 formed, μM/l	27.4	4.6	18.7	16.1	27.4	3.9	7.7
Inhibition, %		83.2	31.8	41.2	0	85.8	71.9

Worm: *P-21*, 1st day of the 5th instar. Mouse liver: 0.26 g.

Table III
Inhibition with Worm Tissue upon Cattle Liver Extract

H donator added	none			acetaldehyde		
Worm tissue added, g	none	0.1	0.05	none	0.1	0.05
NO_2 formed, μM/l	9.4	0.6	2.5	54.8	35.2	41.6
Inhibition, %		93.6	73.4		35.8	24.1

Worm: *P-44*, 5th day of the 5th instar. Cattle liver: 0.5 g.

4) H. Omura, *Sci. Bull. Fac. Agr. Kyushu Univ.*, **14**, 423 (1954).

5) K. Yamafuji and S. Nakamura, *Enzymologia*, **16**, 130 (1953).

TABLE IV
EFFECT OF ALTERATION OF REDUCING ACTIVITY ON INHIBITION

Liver used	mouse						cattle					
Amounts of liver, g	0.1		0.2		0.3		0.1		0.3		0.5	
Worm	*P–21*, 1st day of the 5th instar						*P–44*, 5th day of the 5th instar					
Worm tissue added, g	none	0.3	none	0.3	none	0.3	none	0.1	none	0.1	none	0.1
NO_2 formed, μM/l	6.1	–	21.1	2.1	32.6	4.0	12.8	5.0	37.2	23.4	48.8	34.8
Inhibition, %		100		90.0		87.7		60.9		37.1		28.7

TABLE V
INHIBITION WITH WORM TISSUE UPON GREEN ALGAE

Algae added	intact cell suspension			cell free extract		
Worm tissue added, g	none	0.1	0.05	none	0.1	0.05
NO_2 formed, μM/l	56.0	24.0	43.2	9.9	6.5	9.3
Inhibition, %		57.1	22.9		34.1	6.1

Worm: *P–21*, 1st day of the 5th instar. Green algae: 0.5 g.

TABLE VI
DISTRIBUTION OF THE INHIBITOR IN SILKWORM

Worm	Part	blood	digestive juice	digestive canal	feces
	Strain	*P–44*	*P–21* × *P–44*	*P–21* × *P–44*	
	Age	2nd day of the 5th instar	1st day of the 5th instar	1st day of the 5th instar	
Inhibition, %		3.6	41.4	1.2	88.9

TABLE VII
EFFECT OF VARIOUS CONDITIONS OF WORM TISSUE

Tissue tested	acetone powder		pupae		virus-diseased		nitrite-fed	
Worm	*P–44*, 2nd day of the 5th instar		*P–44*		*P–21*, 5th day of the 4th instar		*P–44* × *P–21*, 2nd day of the 4th instar	
Amount added, g	0.1	0.05	0.1	0.05	0.1	0.05	0.1	0.05
Inhibition, %	20.8	11.5	49.6	33.2	51.0	23.3	89.3	75.8

described in the preceding paper[6]. By the estimation cited in Table V., we have been able to demonstrate that the hindering faculty of larval tissue is effective on reduction with algae, regardless of destruction of cells. In contrast with cattle liver, however, the retardation with worm tissue was greater upon the intact algal cell suspension whose reducing power was stronger than that of the cell-free-extract. At any rate, the inhibition with silkworm tissue was available on nitrate reduction of plant as well as in animal tissue.

Following this, the distribution of the inhibitor in silkworm was investigated by the use of the nitrate reducing system of cattle liver extract and acetaldehyde. Since different strains of worm were used, the relative inhibitions were determined using 0.2 g portions of blood, alimentary canal wall, digestive juice and worm feces respectively. Blood and digestive juice were diluted 10 times with phosphate buffer, heated in boiling water for 10 minutes and devoted to examination. As for the canal wall and feces, their boiled extracts were applied to use. As it can be seen from Table VI., the inhibitor was contained in digestive juice while there was a negligible amount in the canal and blood. However, worm feces were most effective in the hindrance of nitrate reduction.

In our laboratory the dried powder of larval tissue prepared by repeated dehydration with acetone is used for studies on the various enzymes. It has been found that the inhibitor can be retained in powder preserved for half a year after preparation at room temperature, although it seemed to be diminished. The insect suffers a remarkable physiological change in its metamorphosis. However, this inhibitor is successively contained until the pupal stage. In this

6) H. Omura, *Enzymologia*, **17**, 127, (1954)

Table VIII
Inhibition with Precipitates

Precipitate added, g	none	0.6		0.4	
Part		supernatant	coagulated	supernatant	coagulated
NO_2 formed, μM/l	22.6	1.8	22.7	2.2	22.6
Inhibition, %		92.0	0	91.1	0

Mouse liver: 0.4 g.

case, the extract of the whole body of pupae was examined. Confusion of metabolism in the larval body is brought about, when the worm is attacked with polyhedral virus disease or fed with a "virus inducer" such as nitrites or hydroxylamine[7]. Larvae of hybride, *P-44* × *P-21*, were fed with M/15 KNO_2, immediately after awakening from the third sleeping and examined on the following day. As for the viral worm, the tissue of caterpillars previously infected by injection with a diluted alkaline virus solution as usual and had just been affected, was used. The inhibitor was also maintained under these circumstances. The results obtained are presented in Table VII.

Isolation and Identification of the Inhibitor. It has been found that the inhibitor in the larval tissue can be extracted more easily by means of alkaline solution. Therefore, extract of the worm tissue with 2% $NaHCO_3$ solution was employed for study of the inhibitor. By the careful addition of glacial acetic acid to this extract, its pH was adjusted to about 4.2, and the proteins deposited. After standing in an ice-chamber overnight, the precipitates were collected by centrifuge and washed with diluted acetic acid of pH 4.2, from 8 to 10 times, and finally several times with cold water. These precipitates were suspended in a phosphate buffer of pH 6.0, heated in boiling water for 10 minutes, and the coagulated part was centrifuged off. Concerning both the clear supernatant and coagulated parts, inhibiting abilities were examined as in the case of fresh tissue. From the results shown in Table VIII., it is evident that the inhibitor is precipitable at pH 4.2, but differs from the heat coagulated proteins.

When the extract of the precipitate was concentrated, inhibiting ability increased and finally the yellow-brown particles, though in a small quantity, were produced. On the other hand, if the suspension was allowed to stand for a week or more in a refrigerator after first precipitation from the alkaline extract of the fresh tissue, similar particles were also formed in the gray bulky proteinous precipitates. As these particles were remarkably heavier than the other deposites, it was easy to isolate them by the repetition of suspending and decanting or centrifuging. At room temperature, these particles were either insoluble or difficultly-soluble in water and in acid, but quite soluble in alkaline solution. On heating, most of these particles were dissolved, even in almost neutral phosphate buffer. Then, 5 mg of these particles was dissolved in 5 ml of M/15 phosphate buffer by heating and the clear supernatant was devoted to the inhibiting test. As shown in Table IX., even a very small amount of the particles such as 1 or 2 mg, indicated a strong inhibition on nitrate reduction. Therefore, it can be infered that the yellow-brown particles isolated, are nothing else than the inhibitor in worm tissue itself.

Table IX
Inhibition with Particle Isolated

Particle added, mg	none	2.0	1.0	0.5	0.1
NO_2 formed, μM/l	30.8	2.6	3.9	17.0	22.8
Inhibition, %		91.6	87.3	44.8	26.0

Mouse liver: 0.25 g.

The extinction spectrum of the phosphate-solution of this particle, measured with a Beckman spectrophotometer is given in Fig. 1. The phosphate-solution was diluted 50 times its volume with water and then estimated. Therefore, the concentration of this solution was 0.02 mg particles per ml. A glance of Fig. 1 revealed that there were special absorptions in the ultraviolet region, two maxima at 290 and 235 mμ, and a minimum at 260 mμ, and no absorption in the visible-wave length. This spectrum is closely resembles that of uric acid, which is the final excretory-substance of this insect. For the purpose of comparison, the extinction curve of 5×10^{-5} M uric acid is also drawn in Fig. 1. In addition to these particles and pure uric acid, such ultraviolet spectra were estimated on the heat extract of both larval tissue and pupal body, which were used for the tests and showed positive inhibiting efficiencies. In this

7) K. Yamafuji, H. Omura and T. Sakamoto, *Enzymologia*, **15**, 210 (1952).

case, 1 g of each tissue and body was extracted with 10 ml phosphate buffer by heating and optical densities were read, after being diluted one hundred times. Similar absorption curves were obtained as was expected. Moreover, the particle gave a positive Murexide reaction to uric acid. Thus it may be infered that inhibition with worm tissue may be attributed to uric acid. Accordingly, the fact indicating that inhibiting ability is most effective in worm feces and that the inhibitor is not to be decomosed by autolysis is also reasonable. Furthermore, it has often been reported that the tissue of silkworm contains uric acid[8]. From the assumption that the absorption at 290 mμ is due to uric acid, it is estimated that contents of acid in the extracts of the caterpillar tissue and pupal body amount to about 16.3 and 10.3 mg per ml.

The influence of pure uric acid (Merck's reagent) on the nitrate reduction of mouse liver was estimated, in order to confirm the fact whether the inhibitor was uric acid, as it had been demonstrated. While uric acid was difficultly soluble in water, this acid can be dissolved to about 10^{-2} M in phosphate buffer at pH 6.0 by heating in the same manner as the particle isolated. The figures in Table X. assure us that uric acid inhibits nitrate reduction as well as the enzymatic decoloration of methylene blue.

TABLE X

INHIBITION WITH URIC ACID

Conc. of uric acid, M/l	0	10^{-3}	5×10^{-4}	10^{-4}
NO_2 formed, μM/l	45.6	3.8	6.4	36.8
Inhibition, %		91.7	86.0	19.3
Time of M.B. decoloration, min.	11	17	15	11

Mouse liver: 0.4 g.

Of course, no retardation of the colorimetric estimation of nitrites by pure uric acid could be observed, neither activation of nitrite decomposition. Whereas, on the other hand, though the Murexide reaction with uric acid is unique in color, other purines such as xanthine and guanine also show a positive reaction against the same test. Therefore, absorption spectra of these purines were estimated, on the same concentration level. These curves in Fig. 1 give explaination that the inhibitor in worm tissue is uric acid, and not other homologues.

On the other hand, even when proteinous precipitates were removed immediately after first deposition of the alkaline-extract of worm tissue and the supernatant left to stand for a long time in the cold, white crystals were obtained and recrystallized with lithium carbonate. These crystals were also effective in hindering nitrate reduction, as indicated in Table XI.

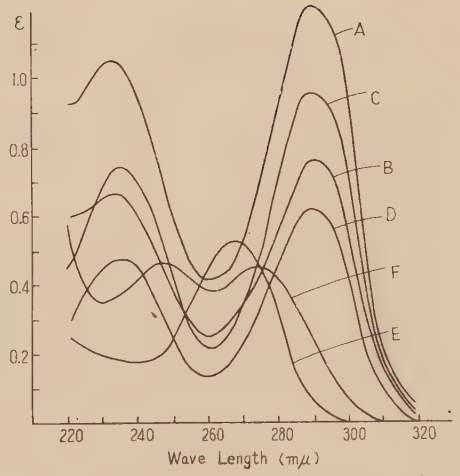


FIG. 1. Absorption Spectra of Extract of larval tissue (A), Extract of pupal body (B), Particles isolated (C), Uric acid (D), Xanthine (E) and Guanine (F).

This crystal was also identified with uric acid on the basis of its Murexide reaction and ultraviolet spectrum. Thus, it is confirmed that uric acid, the main component in excreta of this insect, is itself the inhibitor, in worm. Urea, other excretory substances in animals are, however, inefficient on reduction under such a concentration of uric acid as that which was tested.

The fact that the inhibition of nitrate reduction by worm tissue is due to uric acid is supported by the experiment, shown in Fig. 2. In advance of the nitrate reduction with mouse and fowl liver extracts, 5×10^{-4} M uric acid and the heat-extract of acetone preparation of *P–44*, corresponding to 0.01 g of original powder were preincubated with both livers at 40°C for 2.0, 1.5, 1.0, 0.5 and zero hours, respectively. In the case of mouse liver, the formation of nitrites increased in proportion to prolonged time of

TABLE XI

INHIBITION WITH ISOLATED CRYSTAL

Crystal added, mg	none	1.0	0.5	0.1
NO_2 formed, μM/l	23.8	7.2	11.6	20.4
Inhibition, %		69.7	51.3	14.3

Mouse liver: 0.25 g.

8) S. Shimizu, *Bull. Sericult. Exp. Station*, **11**, 397 (1943).

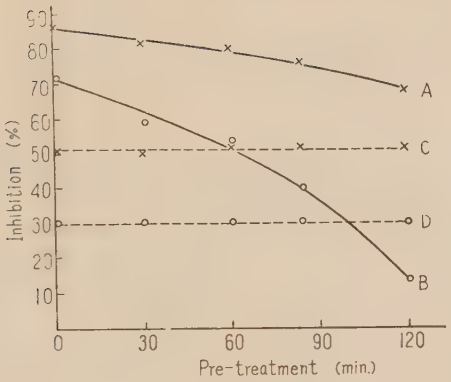


FIG. 2. Effect of Pre-treatment with Mouse- and Fowl Livers upon the Inhibiting Rates of Uric Acid and Worm Extract.

A: Uric acid B: Worm extract treated with mouse liver.
C: Uric acid D: Worm extract treated with fowl liver.

pre-treatment as for both uric acid and the worm tissue extract; that is, inhibiting rates were decreased. On the contrary, retardation in the fowl liver was kept at a constant level. This fact might probably be interpreted as that uric acid is destroyed by the uricase co-existing in mouse liver and inhibition is recovered with degradation of acid, but hindrance is constant in the case with fowl liver because uric acid could not be decomposed owing to the lack of uricase in this liver.

Nitrate Reduction with Silkworm Tissue. In order to explore nitrate reducing ability in the larval tissue, it was necessary to remove the inhibitor without having any undesirable influences upon catalytic activity. For convenience, as already stated, the inhibitor in worm tissue, mostly uric acid, is extracted with diluted alkaline-solution, and nitrate reducing ability is fairly tightly bound to the tissue. Therefore, larval tissue was extracted with 2% $NaHCO_3$ solution and washed with sterilized water and then suspended in phosphate buffer. Nitrite formation was estimated with this tissue suspension. Though it was very weak, the formation of nitrites could be detected. It may be considered, however, that native hydrogen donators and other factors of the tissue could be removed together with the inhibitor by alkaline extraction. By supplementing glutamic acid of 10^{-2} M, increase of the nitrite production was recognized. Furthermore, when boiled liver-extract of 0.1 g a fresh one was added, the activation of nitrate reduction, though considerably slight, was also observed, and the addition of both glutamic acid and liver-extract was found to be most efficient. The results are indicated in Table XII. Therefore, it can be supposed that the ability of nitrate reduction is possessed in the tissue of silkworm but it cannot be detected on account of the inhibiting action of uric acid.

TABLE XII
NITRATE REDUCTION WITH SILKWORM TISSUE

Added,	none	glutamic acid	liver extract	glutamic acid and liver extract
NO_2 formed, μM/l	2.2	9.4	4.8	25.0

Worm: *P-44×P-21*, 4th day of the 5th instar, 0.5 g

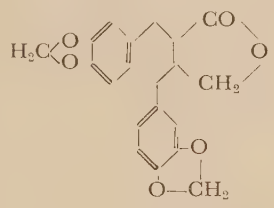
Acknowledgement. I wish to express my hearty thanks to Prof. Dr. K. Yamafuji for his kind guidance and to Mr. H. Takahashi for his generous assistance, throughout this work. This study was supported in part by a Grant in Aid for Scientific Research from the Ministry of Education.

Hibalactone (Savinin) as a Synergist for Pyrethrins and Allethrin

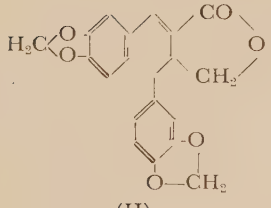
Sir:

In the previous paper[1], the author made a report on the synergistic effect of hinokinin (I) on pyrethrins, and pointed out the necessity of the presence of a γ-lactone ring as a central nucleus for the cause of synergism.

It is an interesting problem to investigate that to what degree the pyrethrins and allethrin are activated by hibalactone (savinin) (II) which has recently been isolated independently by Masumura[2] from "chyabohiba", *Chamaecyparis obtusa* Sieb. et Zucc. in Japan, and by Schrecker et al.[3] from "sabina",



(I)



(II)

Juniperus sabina L. in America, because this compound is identical with hinokinin, both in its structure of the central nucleus and in its steric configuration as shown in (I) and (II).

The author has attempted to clarify the synergistic effect of hibalactone on pyrethrins and allethrin, and made investigations on the knock-down and lethal effectiveness of pyrethroids-hibalactone emulsions against mosquito larvae, *Culex pipiens* var. *pallens* Coq., by means of the petri-dish method which was previously described by the author[4,5], and also, on the knock-down effectiveness of pyrethroids-hibalactone dusts against house fly, *Musca domestica vicina* Macq., by means of the settling dust apparatus method of Nagasawa[6], comparing its synergistic activity with those of hinokinin and piperonyl butoxide.

Pyrethoid-synergist ratio in synergized emulsions and dusts were 1 : 8, such as 0.025 : 0.200 (ppm) in pyrethrins emulsions or 0.05 : 0.40 (%) in allethrin dusts. The degree of synergism (concentration of pyrethroid in pyrethroid only formulation divided by the concentration of pyrethroid in pyrethroid plus synergist formulation; median knock-down time of pyrethroid only divided by the median knock-down time of pyrethroid plus synergist) of the three compounds calculated from the median lethal concentration or median knock-down time by the probit method developed by Bliss, are given in Table I.

Hibalactone acts as a synergist for pyrethrins similarly to hinokinin, and the synergistic activity of the former is more effective than that of the latter in the knock-down test of dusts against house fly, but the former is, on the contrary, less effective than the latter in knock-down and lethal tests of emulsions against mosquito larvae.

Both hibalactone and hinokinin act effectively as a synergist for allethrin, and synergistic activity of the former is more effective than those of the latter and piperonyl butoxide against mosquito larvae, but the former is, on the contrary, less effective than the latter and

1) H. Matsubara, *Botyu Kagaku*, **15**, 21 (1950); **17**, 143 (1952).
2) M. Masumura, *J. Chem. Soc. Japan*, **76**, 423 (1955).
3) A.W. Schrecker and J.L. Hartwell, *J. Am. Chem. Soc.*, **76**, 4896 (1954).
4) H. Matsubara, *Botyu Kagaku*, **18**, 10 (1953).
5) H. Matsubara, *Res. Bull. Faculty of Agr. Gifu Univ.*, **6**, 124 (1956).
6) S. Nagasawa and T. Takano, *Botyu Kagaku*, **15**, 46 (1950).

TABLE I

THE DEGREE OF SYNERGISM OF HIBALACTONE, HINOKININ AND PIPERONYL BUTOXIDE FOR PYRETHRINS AND ALLETHRIN, DETERMINED BY BIOASSAY WITH MOSQUITO LARVAE AND HOUSE FLY

Active ingredient	Synergist	Determined by knock-down effectiveness against: Mosquito larvae — Degrees of synergism	Mosquito larvae — Relative effectiveness	House fly — Degrees of synergism	House fly — Relative effectiveness	Determined by lethal effectiveness against Mosquito larvae — Degrees of synergism	Mosquito larvae — Relative effectivenss
Pyrethrins	Hibalactone	1.70	1.00	1.54	1.00	1.49	1.00
	Hinokinin	1.92	1.13	1.09	0.71	1.62	1.08
	Piperonyl Butoxide	2.92	1.72	6.43	4.17	—	—
Allethrin	Hibalactone	3.50	1.00	3.21	1.00	2.46 ; 2.36	1.00 ; 1.00
	Hinokinin	3.21	0.92	3.69	1.15	1.88 ; —	0.76 ; —
	Piperonyl Butoxide	2.43	0.69	5.99	1.86	— ; 1.89	— ; 0.80

piperonyl butoxide against house fly.

From the bioassay of the synergized pyrethroids emulsions and dusts with hibalactone using two insects, it has been observed that the combination of allethrin with hibalactone; shows a higher order of synergism than the similar combination of pyrethrins with hibalactone furthermore, its synergistic activity for allethrin is 1.65~2.08 times as effective as that for pyrethrins.

By many investigators, it was found that the combinations of allethrin with synergists were in general, less effective than the similar combinations of pyrethrins, but the results shown in the present investigation did not agree with this finding.

The discovery of such unique synergistic activity as exhibited by hibalactone provide us the hope of the research of some more powerful allethrin synergists which possess the methylenedioxyphenyl group. Other synthesized synergists with relation to hibalactone are now being investigated. A detailed observation on the relations between their chemical structures and synergistic activities for the pyrethroids will be described elsewhere.

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On L-Glutamic Acid Fermentation

Sir:

At present, the principal sources of L-glutamic acid are wheat gluten and soybean cake. Steffen's molasse is also used for the isolation of L-glutamic acid. On the other hand, chemical synthesis has been applied to the preparation of glutamic acid; however the product obtained is always the DL-form which have to be separated from each other by rather difficult and expensive methods. A new practical method for the preparation of L-glutamic acid was devised. This method is concerned with the incubation of certain microorganisms in the medium containing glucose, ammonium salts and minerals.

Searches for the microorganisms which are able to accumulate L-glutamate in the medium were carried out. The accumulation of L-glutamate was detected in the cultures of the microorganisms belonging to the following genera: *Micrococcus*, *Bacillus*, *Streptococcus*, *Xanthomonas*, *Pseudomonas*, *Aeromonas*, *Serratia*, *Escherichia*, *Aerobacter*, *Aspergillus*, *Penicillium*, *Mucor* and *Fungi imperfecti*.

Strains belonging to the Genus *Micrococcus* and *Bacillus* were proved to have a powerful ability to accumulate L-glutamate. A strain of *Micrococcus varians*, which showed the highest level of L-glutamate accumulation, was used in the following experiments.

Investigation of the effects of nitrogen sources on the L-glutamate accumulation revealed that ammonium chloride was the best nitrogen source for this purpose.

It was found that L-glutamate accumulation was more effective by submerged culture than by surface culture, and a mild aeration was favourable. Under the anaerobic condition, both the growth of the organism and the accumulation of L-glutamate were slow.

The following medium was used for the production of L-glutamate: 20 g of glucose, 7 g of ammonium chloride, 1 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$ per liter, the pH was adjusted to 7.0, and 0.5% of $CaCO_3$ sterilized separately was added before inoculation. Two hundred ml of this medium was dispensed in a 500-ml Erlenmyer flask and after it was inoculated, fermentation was carried out at 30° by a rotary shaker. The course of L-glutamic acid fermentation is shown in Table I. α-Ketoglutarate which seems to be

Table I

The Course of L-Glutamic Acid Fermentation.

Incubation time (hrs.)	pH	Glucose (μM/ml)	α-K-G	G^H accumulated (μM/ml)	G^H accumulated yield (%)*	L-Alanine (μM/ml)	L-Aspartic acid
0	7.0	116.1	−	−	−	−	−
24	8.5	111.1	+	trace	−	−	−
47	7.9	106.1	−	0.2	0.2	−	−
71	6.1	93.9	+	2.9	2.5	trace	trace
95	5.2	39.4	+	18.1	15.6	+	+
119	5.2	6.7	+	18.2	15.7	2.7	+
144	5.8	3.3	+	19.5	16.8	2.7	+
168	6.0	3.3	+	4.1	3.5	+	+

α-K-G = α-ketoglutaric acid, G^H = L-glutamic acid

*$\frac{\text{L-glutamic acid } (\mu M)}{\text{glucose } (\mu M)} \times 100$

a precursor of L-glutamate was detected in small quantities. L-Glutamate accumulated in appreciable quantities, but it disappeared rapidly followed by the consumption of carbon source. L-Glutamic acid was identified by its melting point and the optical rotation. It melted at 222–223°C and a mixed melting point with an authentic specimen showed no depression. In 2N hydrochloric acid it showed the value: $[\alpha]_D^{20}$ of +31.5°. L-Alanine and L-aspartic acid were also detected in the broth. L-Glutamic acid corresponding to 23% of the glucose supplied was produced in another experiment.

The authors wish to express their thanks to Mr. K. Shōda of our laboratory for identifying the microorganisms used in this study, and to the Ajinomoto Co. Ltd., for the determination of amino acids.

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